The Journal of Laboratory and Clinical Medicine

VOL. 22 JANUARY, 1937 No. 4

CLINICAL AND EXPERIMENTAL

PHYSIOLOGIC CONSIDERATIONS REGARDING THE ETIOLOGY AND NATURE OF CORONARY THROMBOSIS AND ANGINA PECTORIS*

D. E. JACKSON, PH.D., M.D., AND HELEN L. JACKSON, B.S., M.D.,
CINCINNATI, OHIO

IN OUR opinion many acute attacks in which the circulation is especially involved, and which are ordinarily diagnosed as coronary thrombosis, are really not coronary thrombosis at all, but are in reality due to embolism or thrombosis of the pulmonary vessels. These patients may present virtually all of the signs, symptoms and history of coronary thrombosis, but at autopsy no thrombi (or at least no recent thrombi) may be found in the coronaries. And we strongly suspect from our experiments that even when recent obstruction of a coronary artery is found at autopsy this may not have been the real cause of death. For we have seen *(and felt with the hand)* hearts beat for a considerable time after all circulation through the pulmonary vessels had been blocked off by pulmonary embolism. In some of these cases a coronary artery was also completely blocked as shown at autopsy a few moments after the heart had ceased to beat. In these cases death is due to pulmonary obstruction which prevents blood from passing through the lungs and back to the left auricle. If the obstruction is not complete, the patient may slowly recover as more blood is gradually forced through the lungs and the systemic pressure slowly rises. Fatal cases of this type we suspect represent in some instances the group which Levy and Bruenn[1] have recently described under the tentative designation of "acute fatal coronary insufficiency." In some

*From the Department of Pharmacology of the University of Cincinnati College of Medicine.

of these cases it is sometimes noted in the autopsy reports that pulmonary thrombi were found. We believe they would be found in many more cases if they were carefully looked for.

We have produced pulmonary (and coronary) thrombosis by intravenous injection of an extract of lung tissue. The extract is made by washing thoroughly a few ounces of fresh beef lung, passing the material through a meat grinder and then pounding it thoroughly with sand and water in a mortar. The carefully filtered aqueous extract when added in the quantity of about

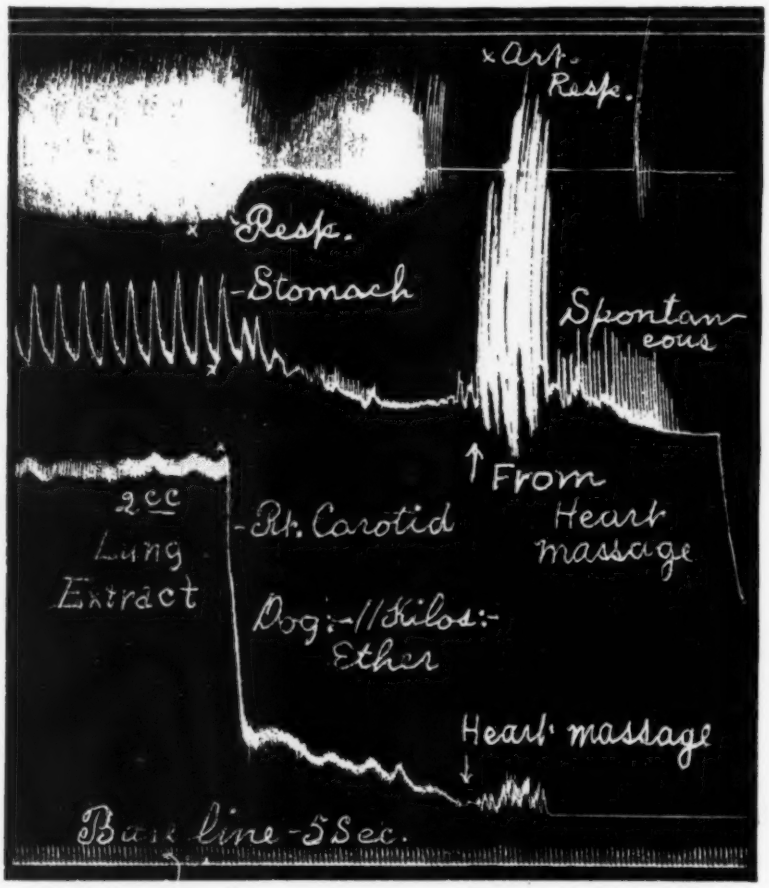

Fig. 1.—The chest and pulmonary circulation were entirely intact in this tracing. In the other tracings the chest was opened. For description see text.

two drops to 5 c.c. of blood freshly drawn through a cannula from the femoral artery (mixed by shaking a few times) will produce within ten to twenty seconds clotting sufficiently firm to permit turning the test tube upside down without spilling any of the blood. Without the extract an equal degree of clotting, under the same conditions, required about four and one-half to five minutes.

When such an extract is injected into a dog's femoral vein, a widely varying range of results may be produced. Within a few seconds the injected

extract is carried through the right heart (where some fibrin or clots may be left; see below) and into the lungs where intravascular clotting and thrombosis may produce results ranging all the way from a barely perceptible rise in pulmonary pressure (with no observable change in the heart and systemic pressure) to almost immediate death. We say "almost" because these deaths are primarily due to failure of the heart to receive blood back from the lungs through the pulmonary veins. At the same time there may be thrombosis of

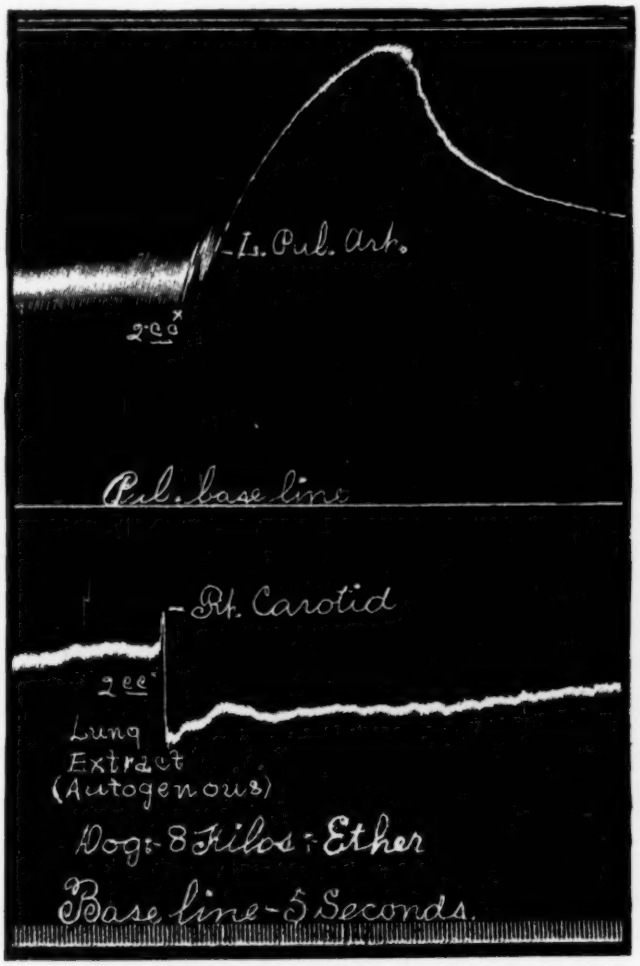

Fig. 2.—For description see text.

one or both of the coronary arteries or of their large branches. We believe that at least in the rapidly fatal cases the coronary thrombosis is not the cause of death, and we suspect that it is not the real cause of death in most cases in which death is delayed for several hours or days with a profound fall of blood pressure lasting through the interval between the acute attack and death. Fig. 1 shows the results produced by an injection of 2 c.c. of lung extract into a dog of 11 kilos. An immediate fall in carotid pressure occurs, because no blood is returned from the lungs to the left auricle. The pul-

monary pressure (not seen in this tracing), however, rises markedly and the right heart dilates but rapidly becomes weaker for no oxygenated blood reaches it from the lungs. The systemic pressure falls very low and clinically the pulse might become imperceptible. The heart beats, however, and perhaps strongly, as one can positively determine by holding it in his hand (with the chest open). With the heart chambers empty, however, the heart must soon fail. In this tracing the early weakening of the respiration is followed by a later increase (as asphyxia quickly develops) with air hunger, gasping, dyspnea, etc. From a long experience with beating hearts in animals, we have no doubt but that death in these cases is usually due to the pulmonary obstruction and not to coronary thrombosis even though one main coronary be completely thrombosed. We should like to emphasize that blocking of a coronary artery in this manner is quite a different matter from performing

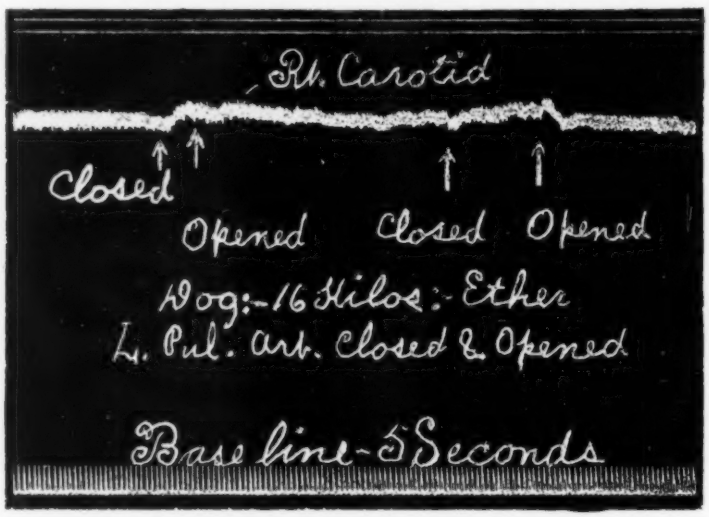

Fig. 3.—For description see text.

an extensive operation and then finally closing off the artery (veins and nerves?) by a ligature. In no instance have we seen ventricular fibrillation produced in experiments of this type, but the animals were always anesthetized, and it might be possible for fibrillation to occur in man in the absence of anesthesia because the vagus and sympathetic reflexes would be much more active. We believe, however, that slow failure of the heart from asphyxia will be the usual outcome.

The size of the dose of lung extract has much to do with the character of the results. A *very small* dose may produce but little effect and the animal quickly returns to its previous condition. Fig. 2 shows the action on the pulmonary and systemic blood pressures of an injection of 2 c.c. (dilute solution) of an autogenous lung extract. A cannula was inserted into the left pulmonary artery and this pressure was recorded by a mercury manometer (the pulmonary pressure is magnified by levers about three times). Thus the left lung was isolated from the circulation and the pressure recorded represented

the changes in the right lung only. A small piece of the left lung was removed, washed, and an extract (dilute) was quickly made of it. On injection (femoral vein), the pulmonary pressure promptly rose and the systemic fell as the result of a certain amount of thrombosis in the pulmonary circulation. But most of the pulmonary circuit remained open, and the animal was able to carry on, with a noticeable lowering, however, of the systemic pressure. We believe many such cases (i.e., with prolonged lowered systemic pressure) occur clinically. We have used the autogenous lung extract in order to make sure that extracts from an animal's own tissues may produce clotting in its own pulmonary vessels.

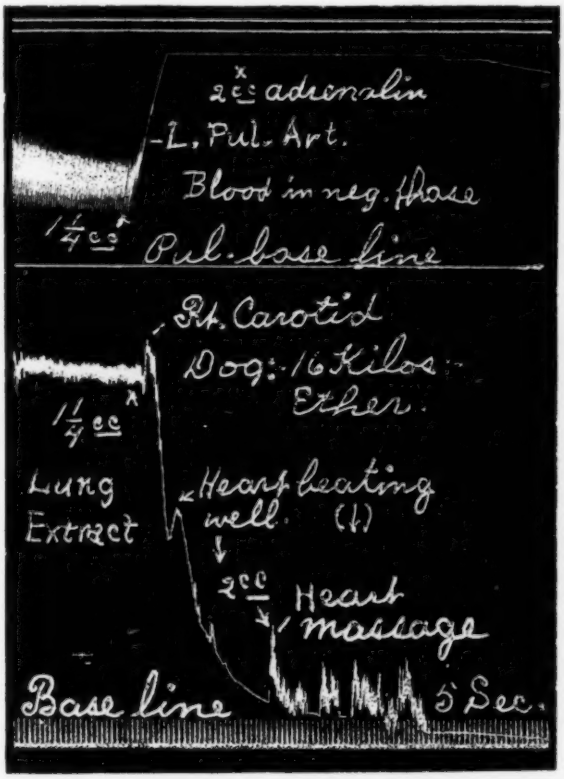

Fig. 4.—Thrombi blocked the whole pulmonary circulation including the neck of the cannula in the left pulmonary artery. For description see text.

When we record pulmonary blood pressure, we insert the arterial cannula into the left pulmonary artery. This leaves only the right pulmonary artery and right lung to carry on the circulation and aeration of the blood. What effect will closing off of one pulmonary artery and one lung have on the systemic circulation? Under normal living conditions, and especially under sudden stress before the animal could become adjusted to such loss, we believe that its resistance would be materially reduced. But we have often closed off the left pulmonary artery (temporarily) without changing the height of the systemic pressure by so much as a millimeter. This means that the circulation can adjust itself almost instantaneously to the loss of half the pulmonary

circuit, or to its return to the circulation. Fig. 3 shows two places (carotid pressure) at which the left pulmonary artery was closed off (with a ligature drawn through a notch in a piece of wood) and then, after an interval, again released. In this tracing there occurred a systemic rise of 2 or 3 mm. of mercury, but that is exceptional. If the technic is *correct* it may be impossible to see any change in the carotid manometer when the pulmonary artery is

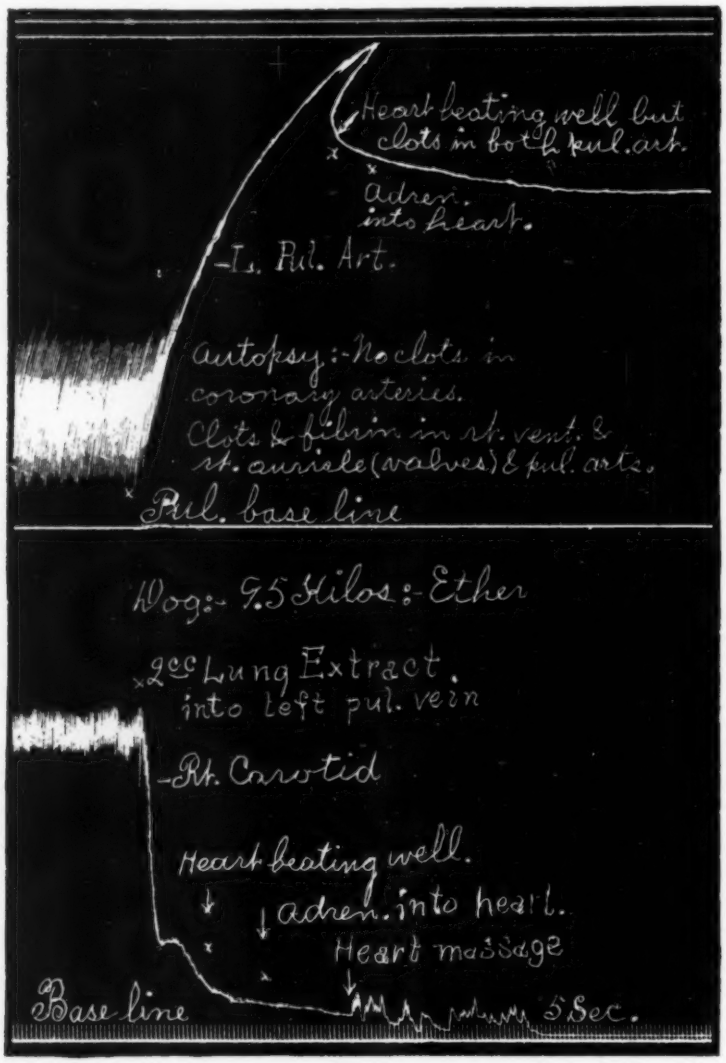
Heart beating well but clots in both pul. art.
Adher. into heart.
-Lt. Pul. Art.
Autopsy:-No clots in coronary arteries.
Clots & fibrin in rt. vent. & rt. auricle (valves) & pul. arts.
Pul. base line
Dog:- 9.5 Kilos:- Ether
2cc Lung Extract into left pul. vein
-Rt. Carotid
Heart beating well.
Adher. into heart.
Heart massage
Base line
5 Sec.

Fig. 5.—For description see text.

closed or opened. This shows that the pulmonary circulation has an enormous factor of safety. But closing off one pulmonary artery raised the pressure in the other pulmonary artery correspondingly. One might suppose that a given dose of lung extract would have a greater blocking effect (by thrombosis) in one lung only than would be the case with both arteries open. This is probably true but the dose is so small in either case that we have usually noted but little difference (see Figs. 1 and 2).

Fig. 4 was made from the same dog as Fig. 3, arrangements having been made in the meantime to record the pulmonary blood pressure. It is seen that injection of a dose of lung extract produced an immediate rise in pulmonary pressure and complete thrombosis of both pulmonary arteries (thus blocking the neck of the pulmonary cannula). The systemic pressure rose for a moment and then fell rapidly, but irregularly, to zero with the heart beating strongly all the while. Adrenalin was injected into the heart and it was also massaged. (Artificial respiration was given throughout the experiment.) The heart could not, however, raise the systemic blood pressure because most of the blood in the body had been blocked off in the lungs and in the great veins, and it could not get back to the left auricle.

A still more striking effect is seen in Fig. 5. In this case 2 c.c. of lung extract was injected into a left pulmonary vein. This injection passed directly through the left auricle and left ventricle and made the complete systemic circuit before returning to the right auricle and right ventricle to go to the pulmonary circulation. The results, however, were quite as marked as in the cases where the extract was injected into the femoral vein. After the systemic blood pressure had fallen almost to zero, the heart was still beating well (but with the chambers empty). Adrenalin injected into the heart (and heart massage) could not cause a rise in pressure. No clots were found in the coronary arteries in this case although the lung extract had passed through the left heart and aorta. We believe, however, from the general tone and vigor of hearts in experiments of this type, even when a marked degree of coronary thrombosis is produced, that the real cause of death is the pulmonary obstruction rather than the coronary thrombosis. We have in no instance found *large clots* in the chambers of the heart in experiments of this character when the autopsy was performed *immediately* after death. We have, however, frequently found small fibrinous clots lodged around the papillary muscles or in the interstices about the base of these, and sometimes in the right auricle. The most striking findings in the heart in these cases is the lodgment of numerous strands and threads of fibrin around and over the chordae tendinae. These threads are perhaps capable in some instances of preventing proper closure of the valves. They would present a queer problem in cases of survival in man.

It has been known for many years that the sudden clotting of a small amount of blood within the vessels renders the remainder of the blood relatively and temporarily incoagulable (negative phase). Melanby has suggested that this may be due to an extensive fibrin formation within the blood, but that the newly formed fibrin is precipitated in a thin layer along the intimal surface of the blood vessels. The blood is thus relatively defibrinated. This is probably often a life-saving process, for a thrombus is certainly a foreign body in a blood vessel, and its constant contact with fresh blood should soon lead to coagulation of the whole blood volume unless some inhibiting influence were at work. In our experimental animals blood drawn from a femoral artery after injection of lung extract (often in very small dosage) does not clot for a long time (perhaps twenty-four hours or more,

or not at all). It is to be expected that in case the animal recovers, the coagulating power of the blood would soon be restored. Yudin[2] in his recent work on collecting and transfusing cadaver blood has virtually corroborated all of these findings in man. He notes that blood taken within six hours after death from the bodies of patients dying of coronary thrombosis, traffic accidents, hanging, angina pectoris, electric shock, etc., does not clot in the usual manner, and in from one-half to one and one-half hours after clotting it undergoes "fibrinolysis" and returns again to the fluid state and will not thereafter coagulate, i.e., the blood passes into the negative phase. We believe these results are due to intravascular clotting as the result of injury which liberates tissue juices into the blood. Yudin surmised that in these patients "fibrinolysis" might begin while the patient is still alive. According to Melanby's hypothesis this would seem rather to depend on how much of the total blood fibrinogen was used up in the initial intravascular clotting, and how much had been reformed by way of recovery. In one of Yudin's accident cases the blood coagulability returned to normal when the patient recovered. At autopsy some hours after John Hunter's death Sir Everard Home noted . . . "the blood not having been completely coagulated, but remaining nearly fluid," and . . . "this evidently arose from the fluid state of the blood." (We cannot conceive how coronary artery spasm could produce these changes in blood coagulation.) We doubt that blood drawn from a patient immediately after the most desperate attack of angina pectoris *with no other accompanying accidents or complications*—but from which a prompt recovery is made, will show this abnormal clotting action. We hope others will investigate this point. In our opinion in cases of this character tissue juices may be liberated into the blood, probably through torn venules or capillaries, as a result of acute, spasmodic, incoordinated contractions of the esophagus or stomach or of rupture of other mediastinal or abdominal structures as a result of these contractions, or, perhaps in some cases from vigorous vomiting, gasping or other movements. More than forty cases of *complete* rupture of the esophagus have been found at autopsy, and we believe that rupture, or tearing of such structures, and especially tearing injuries in the mediastinum or lung tissues, are very likely to liberate such small quantities of tissue extract as may be necessary to cause a pulmonary embolism. And coronary thrombosis should perhaps in some of these cases be considered as an accident in which part, or possibly all, of the thrombi lodged in the coronaries rather than in some other vessels. Lung tissue extract is perhaps more active than that of any other tissue in the body in causing intravascular clotting. Harrison and Mason[3] have recently carried out experiments from which they conclude that tissue extract should be considered the primary cause in the formation of intravascular clots. We agree with this completely.

By new and refined technic Hamilton, Woodbury and Harper[4] have recently shown that during coughing or straining the systolic arterial pressure may suddenly shoot up to an enormous height. They are of the opinion that the intrathoracic and intraspinal pressures are correspondingly raised at the same time and thus the arterial system is protected. We suspect, however,

that in vomiting, during which we believe a certain number of cases of coronary thrombosis (and pulmonary thromboses) are developed, this protective mechanism is inactive, for certainly in some cases when patients vomit the larynx is thrown widely open, a "heaving" sound is made and the abdominal muscles are strongly contracted to force out the stomach contents. Here the sudden rise in systolic pressure could strike the pulmonary and coronary arteries at a time when no compensatory increase in the air pressure in the thorax was present. Moreover in many acute attacks of angina the blood pressure rises considerably as measured by the ordinary cuff and manometer. McCrae[5] refers to a case in which the pressure reached 340 mm. Hg. We suspect that in such cases as this an actual rupture of the walls of a coronary or pulmonary vessel might occur with thrombus formation, and we know that rupture of the heart is occasionally produced. Coronary sclerosis or old infarcts would make bad matters worse. Postoperative and puerperal pulmonary embolism have recently been discussed by Nystrom,[6] and Averbuck[7] has studied the differential diagnosis between pulmonary embolism and acute coronary thrombosis.

It now seems fully demonstrated surgically that in at least certain patients who are subject to bilateral attacks of angina pectoris of approximately equal extent and severity on each side, complete removal of one lobe of the normal thyroid gland[8] is capable of producing immediate, complete, but temporary relief from pain on that corresponding side in attacks of angina which may occur during a period of approximately three to seven weeks immediately following the operation. This lateral relief of pain extends to the midline of the body but not beyond.

Similarly it seems fully demonstrated surgically that in at least some patients of the type indicated above (novocaine or) alcoholic injection (or surgical removal) of the upper five or six thoracic ganglia[9] or section of these posterior roots will produce immediate, complete (temporary or/), and prolonged (or permanent) relief from angina on that corresponding side. The relief extends up to the midline of the body but not beyond. And we may add that removal of the superior cervical ganglion alone[10] may produce complete relief in a certain number of angina patients.

All of these results have been obtained by investigators who believed that the pains of angina come from the heart, or possibly from the coronary arteries, and that the etiology is a spasm of these arteries with resultant anemia (absolute or relative) of the heart muscle.

Let us inquire a little into the mechanism of these heart pains. Shall we presume that the pain of angina pectoris exists as a single unity in the heart, but that it divides into four quarters from either angle of which it may be successfully attacked, when it passes from the heart out into the chest or up into the neck? If the pain in the heart is a unity, then when these sensory nerves pass out of the heart do they divide into two approximately equal divisions each of which reaches the central nervous system along its corresponding side of the body? If so, then alcoholic injection of these fibers *on one side only* should not produce total relief from pain on that side only,

but it should produce relief of half the pain on both sides equally. A mechanism somewhat similar to this is found in the optic tracts. And while motor fibers passing outward to the heart over the vagi or sympathetics may connect with numerous postganglionic neurons and thus distribute these motor effects over a wide area, it does not seem probable that the reverse of this would occur in the case of sensory fibers, i.e., that a small painful spot in the heart could send out painful impulses which would spread to numerous sensory fibers, which, after they pass out of the heart, would divide into two divisions, each passing centrally on its corresponding side of the body, and each capable of being injected with alcohol to relieve the pain on that side of the body only and without any relief on the opposite side. Or shall we suppose that sensory nerves coming from the left side of the heart, or the left coronary artery, transmit pain which is felt in the left side of the body only, and that sensory fibers from the right heart, or right coronary, produce pain in the right side of the body only? As opposed to this we know that occasionally a patient who suffers a severe attack with pain on the left side of the body only, may be shown at autopsy to have thrombosis of the right coronary artery, and the reverse holds true for the left coronary artery and the right side of the body. This would perhaps not quite rule out the possibility that a patient could have a thrombosis in the right coronary and a spasm of the left coronary at the same time. But when a patient has an attack of coronary thrombosis without any pain, and which may only be detected at autopsy long afterward, then one might question the part played by the coronary arteries in stimulating the pain nerves under any circumstances. Or should we suppose that alcoholic injection on one side only, by some obscure process, might put out of action one lateral half only of some "irritable focus" in the cord or medulla? Or that pain nerves for the left side of the body come from the left half of both coronary arteries, and pain nerves for the right side of the body come from the right half of both coronary arteries so that pain in the heart would be equally represented on each side of the body? Perhaps we can best illustrate this problem by referring to the small boy who had lived all his life in town but was permitted one day to visit friends who lived in the country. When he returned home in the evening his father asked him if the people he had visited had butchered hogs that day. The boy reflected for a moment and then replied, "Yes, they butchered a half a hog." When one-half of a bilateral angina pectoris is "butchered" what becomes of the other half? The answer is that it survives, usually becomes worse, and may ultimately lead to the death of the patient.

In an earlier paper[11] to which we must refer we have expressed the opinion that angina pectoris is due to acute, spasmodic, incoordinated contractions of the esophagus and stomach. Obviously pain resulting from these cramplike contractions will be carried up to the central nervous system by the vagi or by visceral sensory branches of the thoracic nerves. And in the main these will be unilateral in distribution and hence subject to unilateral attack. There is also much evidence, both experimental and clinical, to show that sensory fibers in the phrenics may be involved in many cases of angina pectoris.

When the patient compains of "needlelike" pains in the region of the heart, especially at its apex, we suspect that that indicates involvement of phrenic sensory fibers. A most instructive case of this type has been described (with illustrations) by Porter[12] in a case of "diaphragmatic flutter with symptoms of angina pectoris." In this case the "fluttering" movements of the diaphragm (250 or more per minute) were superimposed on the normal respiratory diaphragm movements, and the patient complained of an "agonizing pain in the left pectoral area accompanied by a cramplike pain in the left arm and ring and little fingers of the left hand." The left phrenic nerve was injected with procaine hydrochloride which caused the pain to disappear immediately, the diaphragmatic flutter ceased and the left side of the diaphragm was paralyzed. Seven hours later (when the local effects of the drug had worn off) all of the earlier symptoms recurred and again procaine was injected, but this time into the right phrenic nerve, which resulted in immediate disappearance of the pain, relaxation of the right side of the diaphragm and disappearance of the "flutter." When the local anesthesia wore off, the pain and "flutter" returned. "The heart and aorta were entirely normal." This case is instructive as showing that abnormal movements of the diaphragm, probably in the neighborhood of the central tendon and the cardiac orifice, can lead to painful disturbances that undoubtedly involve exactly the same sensory nerves that carry the pain from the left arm and ring and little fingers in a typical attack of angina pectoris. And the procaine paralysis of the phrenics with immediate relief we believe is practically analogous to the relief obtained by excision of the superior cervical ganglion or of one lobe of the thyroid, but with the exception that these latter cases may involve two mechanisms (and in some instances possibly three) instead of the one motor mechanism involved in paralysis of one phrenic nerve. We believe that these operations in the neck produce a disturbance of the vagus nerve and thus lead to some, perhaps temporary, decrease of its motor action on the esophagus and stomach. If the injury to the vagus nerve is but slight, it may soon regain its former action on the esophagus and stomach and anginal attacks may recur. Also sensory fibers in the vagi may be injured during the operation (as already surmised by others[8]) and thus block off painful impulses which we believe come from the esophagus or stomach or other neighboring structures (but which others have supposed come from the heart). There is also a third possibility: that injured or divided cervical sympathetic fibers whose postganglionic neurons are descending from the superior cervical ganglion to the esophagus or stomach may ordinarily have an inhibitory action on the esophagus or stomach. Section of these fibers might sensitize their myoneural junctions in the esophagus and stomach to adrenalin and thus increase their inhibitory action when conditions of stress tend to increase adrenalin secretion. Permanent relief following (at an interval) total thyroidectomy we believe to be due to a decrease in activity and spasticity of the gastrointestinal tract rather than to a decrease in circulatory activity.

The complex character of the innervation of the esophagus and stomach should be borne in mind. Apparently the vagi carry both motor and inhibitory

fibers and the sympathetics probably also carry both motor and inhibitory nerves (making four sets, unequally distributed) and in addition the vagi carry sensory fibers. In addition to these the local nervous mechanism can act almost independently of the extrinsic nerves although normally these perhaps control most of the movements. Sensory fibers from the thoracic nerves are also distributed to this region if they do not actually penetrate the walls of the esophagus and stomach. Recently McSwiney[13] and his coworkers in England have described a new group of sensory fibers running *in the vagi from below the diaphragm* up into the thorax where they leave the vagi, pass back through the mediastinum to the posterior root ganglia and thence up the cord to the brain. Their stimulation (readily carried out mechanically) below the diaphragm causes dilatation of the pupil when all other nervous connections have been severed. Presumably fibers of this group may transmit pain from below the diaphragm and from areas within the chest.

Within very recent years the pain of angina has been supposed to be due to the accumulation of lactic acid in the heart muscle as the result of local ischemia. Recently Evans[14] and his coworkers in England, in at least two publications, have shown that, in contrast to skeletal muscle, the working heart readily utilizes lactic acid which not only serves as a food more readily assimilable than dextrose but also slows and strengthens the heartbeat, increases cardiac contractility and efficiency, dilates the coronary arteries and increases the coronary blood flow. "The significance of this phenomenon is of particular interest, since it is exhibited only by the heart," and also "it seems highly probable that the effect is specific to the lactate ion."

CONCLUSIONS

1. In our opinion many acute attacks now diagnosed as coronary thrombosis (which may or may not be present) are really due to pulmonary embolism (or pulmonary thrombosis). We believe that usually juices liberated from tissues injured during anginal attacks, vomiting, straining, etc., cause the intravascular clotting resulting in dyspnea, cyanosis, and circulatory and blood coagulating disturbances.

2. The lungs serve as a filter for the general circulation and are more liable to collect emboli than are the coronary arteries. The paper discusses the general relationships of circulatory and respiratory disturbances resulting from pulmonary or coronary thrombosis produced experimentally.

3. The nature of the pain developed in angina pectoris and coronary thrombosis, the nerves involved, and the information revealed by surgical, pharmacologic, or physiologic investigation of these mechanisms are discussed.

4. Attention is called to the inconsistencies between the latest findings on the utilization of the lactate ion by the heart, and the local anemia (with lactic acid formation) by which current hypothesis attempts to explain the pain in angina pectoris (by coronary arterial spasm). There is no clinical or physiologic evidence whatever to indicate that a pain-producing spasm of the coronary arteries ever occurs.

REFERENCES

1. Levy, Robert L., and Bruenn, Howard G.: Acute Fatal Coronary Insufficiency, J. A. M. A. **106**: 1080, 1936.
2. Yudin, S. S.: Transfusion of Cadaver Blood, J. A. M. A. **106**: 997, 1936.
3. Harrison, Stearley P., and Mason, Edward C.: A Study of Intravascular Coagulation, Am. J. Physiol. **116**: 71, 1936.
4. Hamilton, W. F., Woodbury, R. A., and Harper, H. T.: Physiologic Relationships Between Intrathoracic, Intraspinal and Arterial Pressures, J. A. M. A. **107**: 853, 1936.
5. McCrae, Thomas: Osler's Principles and Practice of Medicine, ed. 12, New York and London, 1935, D. Appleton-Century Co., p. 853.
6. Nystrom, Gunnar: Lectures on Embolism and Other Surgical Subjects, Baltimore, 1936, Williams & Wilkins Company (The Abraham Flexner Lectures).
7. Averbuck, Samuel H.: The Differentiation of Acute Coronary Artery Thrombosis From Pulmonary Embolization, Am. J. M. Sc. **187**: 391, 1934.
8. Weinstein, A. A., Davis, David, Berlin, D.D., and Blumgart, H. L.: The Mechanism of the Early Relief of Pain in Patients With Angina Pectoris and Congestive Failure After Total Ablation of the Normal Thyroid Gland, Am. J. M. Sc. **187**: 753, 1934.
9. White, James C.: The Autonomic Nervous System—Anatomy, Physiology and Surgical Treatment, New York, 1935, The Macmillan Co., p. 242.
10. Coffey, W. B., and Brown, P. K.: The Surgical Treatment of Angina Pectoris, Arch. Int. Med. **31**: 200, 1923.
11. Jackson, D. E., and Jackson, Helen L.: Experimental and Clinical Observations Regarding Angina Pectoris and Some Related Symptoms, J. Lab. & Clin. Med. **21**: 993, 1936.
12. Porter, William B.: Diaphragmatic Flutter With Symptoms of Angina Pectoris, J. A. M. A. **106**: 992, 1936.
13. Harper, A. A., McSwiney, B. A., and Suffolk, S. F.: Afferent Fibers From the Abdomen in the Vagus Nerve, J. Physiol. **85**: 267, 1935.
14. Bogue, J. Yule, Evans, C. Lovatt, and Hsu, F. Y.: The Significance of Lactic Acid Uptake by the Mammalian Heart, J. Physiol. **84**: 55, 1935. Also, Quart. J. Exper. Physiol. **24**: 347, 1935.

STUDIES ON THE PHYSIOLOGIC ACTION OF DI-ETHYLENE GLYCOL*

I. The Effect Upon the Irritating and Toxic Properties of Cigaret Smoke

H. B. Haag, M.D., Richmond, Va.

IN ORDER to insure a satisfactory moisture content and to facilitate manufacture, some hygroscopic agent is customarily added to cigaret tobaccos. Glycerin has long been used for this purpose. In several recent publications it has been indicated that smoke from cigarets containing di-ethylene glycol is less irritating than smoke of cigarets made with glycerin or even devoid of any added hygroscopic agent. Such, for instance, were the observations of Mulinos and Osborne,[1, 2] who prepared solutions by drawing smoke from the various types of cigarets through a small volume of physiologic salt solution, water, oil, or Ringer's solution, and then noted the resulting irritation when these smoke solutions were instilled into the conjunctival sac of rabbits. These authors chose as a criterion of irritation, the edema-provoking properties of the smoke solutions, and found the solutions made from di-ethylene glycol-impregnated cigarets to be least irritating. Flinn[3] similarly concluded from clinical trials that the

*From the Department of Pharmacology, Medical College of Virginia.

smoking of di-ethylene glycol-treated cigarets seldom caused irritation; indeed, it actually appeared, generally to be followed by an ameliorating effect upon an irritated throat and tongue. These later results are somewhat at variance with the laboratory findings of Mulinos and Osborne,[1, 2] who did observe irritation of some degree in the majority of the experiments upon di-ethylene glycol-treated cigarets. A discordant note is sounded by Sharlit,[4] who, after clinical studies, was unable to report any difference between the irritating properties of cigarets made with glycerin or di-ethylene glycol.

Neither Mulinos and Osborne[1, 2] nor Flinn[3] ventured a definite explanation to account for the differences in the degree of irritation noticed for the several types of cigarets. However, Flinn[3] states that "acrolein does not enter into our discussion as it is found in all cigaret smoke no matter what hygroscopic agent is present."

The practical importance attached to the observations mentioned prompted the present report which embodies comparative experimental studies upon the

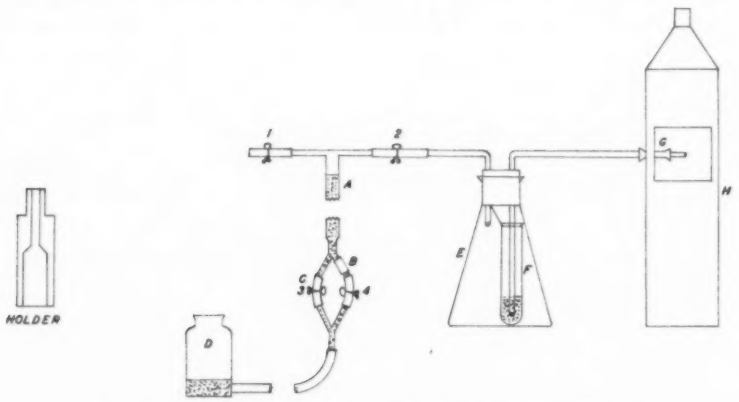

Fig. 1.—Smoking apparatus for irritation experiments.

local irritating effects of smoke solutions prepared from cigarets containing either glycerin or di-ethylene glycol as hygroscopic agents. We employed, as far as possible, the general technic of Mulinos and Osborne.[1] The toxicity of smoke solutions from the two types of cigarets was also determined.

Two identical blends of tobaccos were prepared with the exception of the hygroscopic agent employed. Sample "A" contained 2¾ per cent di-ethylene glycol, as verified by analysis; sample "B," 3 per cent glycerin, cigarets of similar concentrations having been used by Mulinos and Osborne.[1, 2] Sufficient tobacco was used to insure complete blending, and the blends and cigarets were manufactured under standard factory conditions.

An attempt was made to duplicate the smoking arrangement of Mulinos and Osborne,[1] but due to several disadvantages, a somewhat different apparatus was employed, the description and advantages of which will be indicated below.

The apparatus (Fig. 1) is composed of 3 sections: the puffing mechanism, the absorption apparatus, and the cigaret holder and draft shield.

The puffing mechanism consists of a 50 c.c. buret, *A*, with a T-tube sealed at the top, and a Y-tube at the bottom. The constricted tube *B* controls the rate

of fall of the water column in the buret. The by-pass *C* permits the rapid return of the water from the leveling bottle *D*, to refill the buret.

Tube *F*, enclosed in a 500 c.c. Erlenmeyer flask *E*, contains the absorbing medium, with an ammonia tube to bubble the smoke through the solution. "*F*" is suspended from the stopper by a thread.

Cigaret holder *G* is of bakelite, machined for easy connection with rubber tubing, and reamed to receive a cigaret without deforming it in any way. "*H*" is a cylindrical can open at the top and bottom with two windows of transparent celluloid. The front window is removable for lighting the cigaret.

To operate, the cigaret marked at a chosen point is inserted into the holder, sealed with a glucose concentrate, and connected to the apparatus. The glucose concentrate is a clear glucose syrup evaporated in vacuo to a consistency that will not wet the cigaret paper. With the window open, pinchcocks *1*, *3*, and *4* closed, and *2* open, the cigaret is lighted by holding a match flame to the end and opening pinchcock *4*. The water column is allowed to fall to the desired volume, and pinchcock *4* closed. The window in the draft shield is closed, and the puffing apparatus prepared for the next puff. This is done by closing pinchcock *2*, opening *1*, raising leveling bottle *D* above the *O* mark on the buret, and opening *3*. When the *O* level is reached, *3* is closed, and *D* returned to the table. Pinchcock *1* is closed and *2* opened. The manipulation of cocks *1* and *2* must be as specified to preserve the slight hydrostatic pressure in *F*. Smoking is continued until the mark on the cigaret is reached.

The superiorities of this apparatus, although requiring manual operation, are as follows:

Any desired volume of puff is easily reproducible within small limits (± ½ c.c. or better).

The frequency of puffing is constant for any selected interval.

The duration of the puff is maintained constant within a fraction of a second by the jet in the water line, but may be varied by exchanging jets.

The decreasing suction as the water column falls reproduces the tendency in human smoking to a strong draft at the beginning of a puff, and diminishing as the mouth becomes filled.

All cigarets were selected of a uniform weight (1.09 gm. ±0.02 gm.), standard in physical properties, and of the same normal moisture content. Five-sixths of each cigaret was smoked in a conditioned laboratory atmosphere at 85° F. and 45 per cent relative humidity. In keeping with the technic of Mulinos and Osborne,[1] a puff of 25 c.c., lasting four seconds, was taken at fifteen-second intervals, the average cigaret being consumed by 32 puffs, or in eight minutes. The smoke was drawn through 3 c.c. of physiologic salt solution. The smoke solutions of the 2 types of cigarets were prepared simultaneously and employed experimentally the same day.

The smoking technic of Mulinos and Osborne might be considered at fault in several particulars. The rate described in no way approximates that of average human smoking, which should be one of the first aims of any smoking technic. It was found impossible with our apparatus, where the volume and rate of puffing are quite precise, to get 60 puffs from a single cigaret smoked at the rate and volume stated. Again the draft shield used by Mulinos and

Osborne tended to confine an atmosphere of side stream smoke about the glowing tip, inhibiting, if not preventing, natural combustion. This may account in part for the larger number of puffs obtained. Further, if the cigarets were tightly screwed into holders of the commercial type, with a tapered opening, the volume of the puff which was measured might not represent the actual volume of smoke pulled through the cigaret because of leakage and increased resistance at the compressed end. Neither the weight of the cigarets used nor the length smoked is stated in their publications.

While the absorption technic of Mulinos and Osborne was followed in this work, a knowledge of the nature of cigaret smoke reveals its relative inefficiency. Smoke consists of particles of a colloidal nature, dispersed in a true vapor and gas phase. The very soluble portion of the vapor phase is readily absorbed in a bubbler, but the difficultly soluble vapors and colloidal particles pass through almost without absorption. The method of allowing the smoke to completely settle out over water ensures much better extraction. That either type of aqueous solution represents, however, only a part of the total irritating frac-

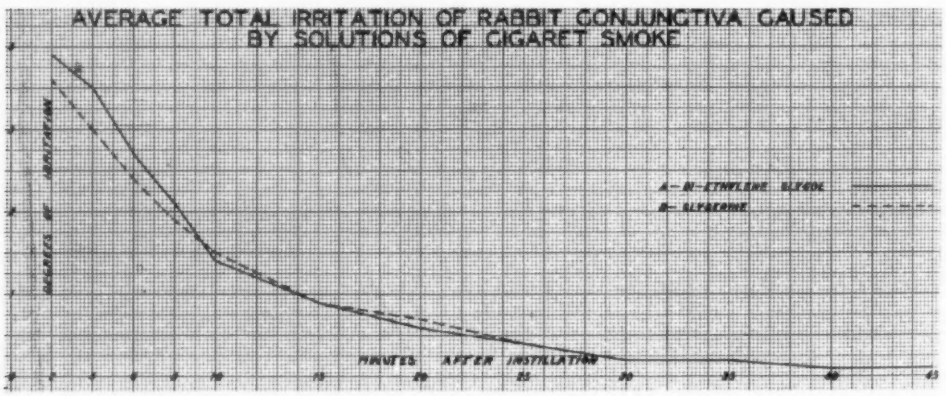

Chart 1.

tion of the smoke may be demonstrated by directing a few puffs of the whole smoke stream against the conjunctiva of the rabbit, and making suitable comparisons with corresponding water solutions.

The rabbits used were fed a diet of Purina Rabbit Chow for a week prior to their use, and were employed only once. In performing the animal observations, 3 drops of the smoke solution were instilled into the left eye of the rabbit, the right eye serving as a control. Because of the fact that different varieties of rabbits occasionally showed differences in the sensitivity of the conjunctiva, 2 rabbits of the same kind were used simultaneously; one for the "A" solution and the other for the "B." The animals were kept in suitable rabbit boxes to facilitate inspection, and after the solutions had been dropped into the eyes, observations were made with the aid of suitably placed lamps and a hand glass of 2 diameter magnification. The observer was at no time aware of which solution was being instilled.

As evidence of the degree of irritation provoked, the objection of the animal, and the blepharospasm, as well as the extent and duration of the hyperemia and

edema of the conjunctiva were tabulated at regular intervals of several minutes. No difference was noted between the two types of smoke solutions as judged by the objection of the rabbit or by the intensity and duration of the blepharospasm. Again, those isolated instances (about 15 per cent) in which definitely discernible macroscopic edema appeared were equal as to incidence, intensity, and duration of edema for both smoke solutions. The hyperemia, which was omnipresent, lent itself particularly well to schematic exhibition both as to duration and intensity, and hence the various stages of this cardinal criterion of irritation were arranged for graphic presentation. The hyperemic condition of the conjunctiva on the upper and lower lids, upper sclera, and nictitating membrane was

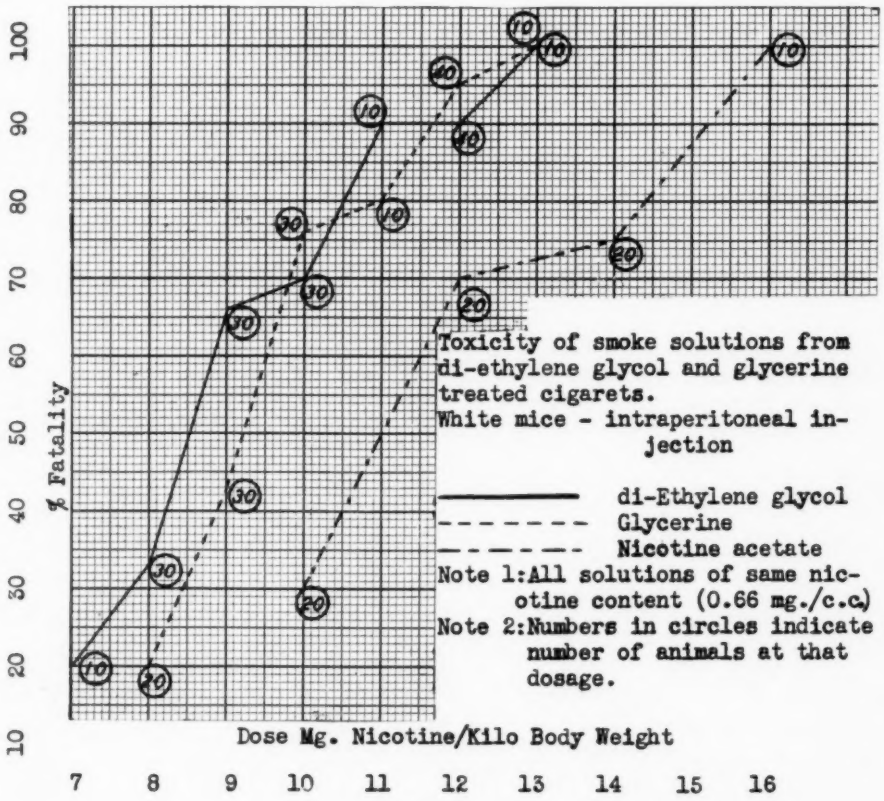

Chart 2.

noted and the degree of irritation observed at any one interval was given a numerical value of from 0 to 2, divided into fractional intermediates. This classification embraced clinical intensities of hyperemia of from "least perceptible" to the maximum observed. Numerical equivalents were then determined for an observation by adding the values given to the four conjunctival areas examined. Chart 1 illustrates the results obtained upon 50 rabbits equally divided for use with the 2 types of smoke solutions.

It appears from our results, based upon four types of observations, i.e., objection, blepharospasm, hyperemia, edema, that there was no significant difference, either as to intensity or duration, in the irritation produced by the instillation into the rabbit eye of the 2 smoke solutions studied.

For toxicity studies smoke solutions were prepared from the 2 types of cigarets by gravity extraction over water. The finished solutions contained the same amount of nicotine, namely 0.66 mg. per c.c., and the toxicity of these solutions, after the addition of enough sodium chloride to make them isotonic, was established upon white mice by intraperitoneal injection. Chart 2 lists the results obtained as compared with control experiments with a solution of nicotine acetate of the same concentration. It is interesting to note that these smoke solutions were slightly more toxic than their nicotine content indicated.

CONCLUSIONS

When aqueous smoke solutions, obtained from di-ethylene glycol-treated and glycerin-treated cigarets, were instilled into the conjunctival sac of rabbits, no differences in the irritating properties of the two types of cigarets were observed, as judged by the appearance of hyperemia, edema, blepharospasm, and the objection of the animal. Likewise, smoke solutions prepared from the two types of cigarets were found to have the same toxicity upon white mice by intraperitoneal injection.

REFERENCES

1. Mulinos, M. G., and Osborne, R. L.: Pharmacology of Inflammation: III. Influence of Hygroscopic Agents on Irritation From Cigarette Smoke, Proc. Soc. Exper. Biol. & Med. **32**: 241, 1934.
2. Mulinos, M. G., and Osborne, R. L.: Irritating Properties of Cigarette Smoke as Influenced by Hygroscopic Agents, N. Y. State J. Med. **35**: 590, 1935.
3. Flinn, F. B.: Some Clinical Observations on the Influence of Certain Hygroscopic Agents in Cigarettes, Laryngoscope **45**: 149, 1935.
4. Sharlit, H.: Cigarette Smoke as a Health Hazard, N. Y. State J. Med. **35**: 1159, 1935.

TULAREMIA DIAGNOSED BY "ROUTINE" BLOOD CULTURE*

N. H. Lufkin, M.D., and A. E. Evenson, B.A., Minneapolis, Minn.

AMONG the notable characteristics of *Pasturella tularensis* is the difficulty of its cultivation on ordinary mediums. McCoy[1] had thoroughly investigated the bacteriology and pathology of tularemia in ground squirrels long before he was able to cultivate the organism. Francis[3] in 1928 reported that the cystine requirement of the organism in culture mediums was one of its unique features. In a case of plague Levin[2] was recently able early to rule out tularemia because the organism grew well on ordinary mediums. The standard procedure for recovering *Pasturella tularensis* from clinical cases of tularemia is the inoculation of a guinea pig with suspected human blood or tissue, and the inoculation of cystine agar with material from lesions found in the guinea pig. Isolation of the organism directly from human tissues was first accomplished by Simpson[4] in 1928, when he inoculated cystine agar with scrapings from the wall of a tularemic abscess.

*From the Department of Pathology, University of Minnesota Medical School, and the Clinical Laboratories of the Minneapolis General Hospital.

Received for publication, March 13, 1936.

An extensive review of the literature has revealed only one case in which *P. tularensis* was isolated on a "routine" medium. This was the case of Gundry and Warner[5] in 1934. During life, blood was inoculated into veal brain broth and into dextrose broth. In seventy-two hours, growth had been obtained in both of these mediums, more luxuriant in the veal brain broth. In this case the diagnosis of tularemia was suspected from the clinical history. It is felt that the following cases merit report because they represent only the second instance in which *P. tularensis* has been cultivated directly from the patient's blood on a routine artificial medium, and because they represent the first cases in which the first suspicion of the disease was aroused through examination of routine blood culture.

CASE REPORTS

The cases were those of a husband and wife admitted to the Minneapolis General Hospital on Oct. 30, 1933.

CASE 1.—The husband, L. R., gave a history which apparently had no bearing on his present illness. He complained of gastrointestinal disturbances, dyspnea, chest pains, and epigastric distress of some fifteen years' duration. He listed a large number of other complaints which were apparently unrelated to the present illness, and he stated that he was unable to tell when his febrile attack had begun, for he had become progressively more ill during the preceding three months.

The temperature on admission was 103° F., pulse 87, respirations 20, and blood pressure 120/70. An expiratory wheeze was heard over the entire chest, but there were no râles. The heart sounds were distant. The abdomen was distended and obese and generally tender. Other physical findings were essentially negative.

The urine contained 2-plus albumin, many casts, 1 to 2 red blood cells, and 2 to 4 pus cells per high power field. The hemoglobin and red counts were normal. The leucocyte count was 5,700 with 87 per cent polymorphonuclear leucocytes, 12 per cent lymphocytes, and 1 per cent monocytes. A roentgenogram of the chest revealed left ventricular enlargement of the heart, and a fullness in the hilus region of the left lung suggesting a glandular or tumor mass. The lung fields were otherwise clear. Repeated four days later, a roentgenogram of the chest showed an early pneumonic process in the left upper lobe, with a cystic area of rarefaction suggesting incomplete consolidation or abscess.

The day after admission there were four large watery stools, and for the next two days diarrhea continued with five to seven stools per day. Repeated cultures of the stools were negative for pathogenic organisms. The temperature remained elevated between 103° F. and 105° F. The pulse rate increased to 150. The patient became cyanotic and died on the sixth day of hospital residence. Autopsy was not done.

CASE 2.—Mrs. L. R. stated that on October 25 she had had a severe chill followed by perspiration. Since then she had had four to five chills daily with weakness, malaise, anorexia, and pain over the bladder. The clinical clerk recorded that the patient had cut her thumb one week previous to admission and had suffered since then from soreness in the left axilla. This almost diagnostic fact was apparently overlooked by internes and staff members.

The temperature was 104.4° F., pulse 90, respirations 24, and blood pressure 120/60. The right cervical and left axillary lymph nodes were enlarged and tender. Other lymph nodes were normal. The chest was without special findings. There was pain and tenderness over the bladder, but the abdomen was otherwise normal. Note was made of an infected wound in the left thumb. Again the significance of this important finding was entirely overlooked.

The centrifuged urine contained 80 to 90 red blood cells per high power field but was otherwise normal. The hemoglobin and red count were normal. The leucocyte count was 8,800. The Wassermann test was negative. A roentgenogram of the chest revealed changes suggesting an early pneumonic process in the right lower lobe.

For twelve days the temperature remained elevated, ranging between 100° F. and 103° F. After four days of normal temperature there were seven days of fever varying between 99° F. and 101° F. Thereafter the temperature remained normal until discharge. Pain over the bladder persisted with exacerbations until December 11. There was a purulent drainage from the infected thumb until November 23. A rash appeared on the hands, arms, and back, and persisted for about ten days. The patient was discharged on Jan. 6, 1934, after two months and one week of hospitalization.

BACTERIOLOGIC AND SEROLOGIC STUDIES

Because of the high fever with relatively low leucocyte counts on admission, typhoid fever was suspected in both of these patients. On the day after admission blood cultures were taken using 20 c.c. of blood in 100 c.c. of 0.3 per cent dextrose beef broth (pH 7.4). Widals were negative. On the second day of residence, the Widals were repeated and agglutinations for paratyphoid were

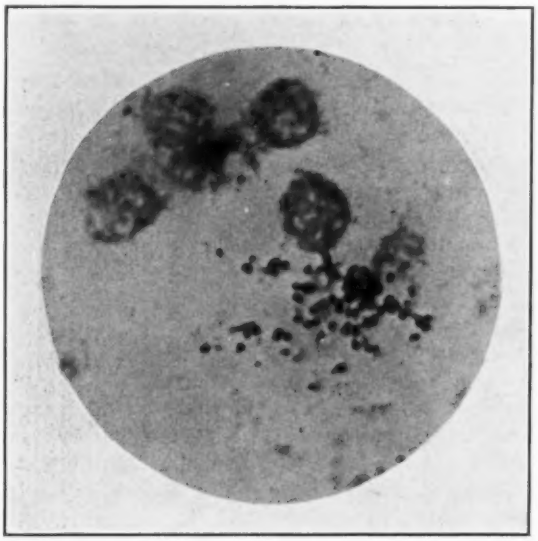

Fig. 1.—Photomicrograph of *P. tularensis* in dextrose beef broth blood culture after seven days. The minuteness of the organism may be judged by comparison with the degenerating erythrocytes in the field. Definition has been greatly sharpened by photographic reproduction.

also done. All of these tests were negative. On November 7 blood culture of the husband showed an abundant growth of extremely minute pleomorphic gram-negative organisms (Fig. 1). Transplanted to blood agar plates these grew scantily and very poorly, but in three days a number of minute, transparent, hemispherical colonies were found. These showed the organisms observed in the original blood culture. Immediately tularemia was suspected.

A guinea pig was inoculated from the blood culture. The next day the pig was sick, and on the fourth day it was dead. At autopsy it showed the typical lesions of tularemia: miliary foci of necrosis in the spleen and to a less extent in the liver. At this time transplants were made from the guinea pig tissues and from the original culture to cystine agar with resulting typical "buttery" growths. On November 14, the wife's blood serum was tested against *P. tularensis* antigen. Agglutination was complete in even the high dilution of 1 to 2,560. Serums from both patients taken on admission for Widals had been

kept. These specimens were tested with *P. tularensis* antigen, and neither that of the husband nor that of the wife produced agglutination. Tested against *B. abortis* antigen, neither the serums taken from both patients on admission nor that of the wife taken on November 14 showed agglutination.

The culture obtained from the husband's blood remained positive for many days, and continued to grow well in successive subcultures on dextrose broth until it was discarded. That from the wife remained persistently negative, and efforts to isolate an organism from it by animal inoculation and transfer to mediums containing cystine were entirely unsuccessful.

FURTHER CLINICAL HISTORY

When the first blood culture examinations suggested tularemia, the wife was interviewed. She gave a vague history of eating wild rabbits and squirrels. Later the son was questioned. He said that on October 22 the husband had killed several wild rabbits which were prepared and eaten on the same day. On October 25 both husband and wife complained of weakness, malaise, and fever, and the husband complained, in addition, of severe pain in the chest. On October 27 the wife first called to the attention of her son a festering sore on her left thumb. She stated at that time that she thought she had cut herself.

SUMMARY

Two unsuspected cases of tularemia in a husband and wife infected simultaneously were diagnosed by routine blood culture, using 20 c.c. of blood in 100 c.c. of 0.3 per cent dextrose beef broth. Blood culture taken from the husband on the seventh day of illness was found to be positive for tularemia seven days later, two days after the death of the patient. Blood culture taken from the wife on the seventh day of illness remained sterile. Serum was taken from both patients on the sixth day of illness. Agglutination tests for tularemia on this serum were negative. Agglutination tests on the serum of the wife taken on the twenty-first day of illness were strongly positive. A guinea pig inoculated from the blood culture of the husband died four days later with the typical lesions of tularemia.

COMMENT

It is unlikely that tularemia will yield positive blood cultures in mediums without cystine except when the infection is overwhelming. Yet it is probable that a greater familiarity with the appearance of *P. tularensis* will result in its more frequent isolation. The organism is so minute and pleomorphic that it might easily be overlooked by those not familiar with it, or searching for it. In our case it is quite likely that we had overlooked the organisms several times before we noticed them. Even then we were uncertain of their nature until after transplantation and animal inoculation. In this connection it is interesting to quote from McCoy's original report:[1] "In many cases the smears show, especially in the leukocytes, fine dust-like objects which are not commonly seen in smears from the tissues of animals dead from other causes, although sufficient control work has not been done. At present we do not attach much significance to these objects, but they will be investigated more fully. Numerous very

small, solid staining bacilli have been seen on a number of occasions. We cannot say at present what importance should be attached to them." No doubt McCoy was actually observing *P. tularensis.*

CONCLUSIONS

1. Quite possibly *P. tularensis* in culture has been overlooked in the past owing to its extreme minuteness and pleomorphism.

2. "Negative" cultures from patients with clinical sepsis should be transplanted at least to blood agar, and should be inoculated into animals.

REFERENCES

1. (a) McCoy, George W.: A Plague-Like Disease of Rodents, Pub. Health Bull. **43**: 53, 1911; (b) McCoy, George W., and Chapin, Charles W.: Further Observations on a Plague-Like Disease of Rodents, With a Preliminary Note on the Causative Agent, Bacterium Tularense, J. Infect. Dis. **10**: 61, 1912.
2. Levin, William: Tularemia and Its Occurrence in Oregon, Northwest Med. **34**: 161, 1935.
3. Francis, Edward: Summary of Present Knowledge of Tularemia, Medicine **7**: 411, 1928.
4. Simpson, Walter M.: Tularemia (Francis' Disease): A Clinical and Pathological Study of Forty-Eight Fatal Cases and One Rapidly Fatal Case With Autopsy, Occurring in Dayton, Ohio, Ann. Int. Med. **1**: 1007, 1928.
5. Gundry, Lewis P., and Warner, C. Gardner: Fatal Tularemia: Review of Autopsied Cases With Report of a Fatal Case, Ann. Int. Med. **7**: 837, 1934.

MINNEAPOLIS GENERAL HOSPITAL

STUDIES ON BARBITURATES. XVI*

BARBITURATE POISONING TREATED WITH PICROTOXIN

WILLIAM S. MURPHY, M.D., HAROLD V. CONNERTY, M.D., ALOYSIUS J. CONNOLLY, M.D., AND THEODORE KOPPANYI, PH.D.,
WASHINGTON, D. C.

DURING the past fifty years several important animal experiments have been performed dealing with the pharmacology of picrotoxin, indicating that it is a medullary drug and stimulates respiration and circulation in depressions caused by different aliphatic narcotics. By far the best and most extensive studies on the antidotal effect of picrotoxin in acute barbiturate poisoning were carried out by Maloney, Fitch and Tatum,[1] and Maloney and Tatum.[2] They tested the antidotal effects of a number of central stimulants including picrotoxin in rabbits poisoned with different barbituric acid derivatives. They found that picrotoxin was the most efficient antidote for increasing the percentage recovery of the poisoned animals and for shortening the recovery time after barbiturate narcosis.

While a number of central stimulants including strychnine, caffeine, coramine, and cardiazol are being used with increasing frequency in human

*From the Department of Pharmacology and Materia Medica and The Department of Pathology, Georgetown University School of Medicine.
Received for publication, March 14, 1936.

barbiturate poisoning, there was only one reported case which has been treated with picrotoxin (Arnett[3]). We were prompted to use this drug clinically not only on the basis of Maloney and Tatum's excellent and convincing researches, but also as a result of our own animal experiments. In these studies we have explored the extreme limits of the antidotal magnitude of picrotoxin, and also analyzed the physiologic phenomena underlying the picrotoxin-barbiturate antagonism. Two preliminary reports have already been published on this subject.[4, 5]

REPORT OF CASES

A. Mrs. F. B., sixty-eight years of age, white, was admitted to Georgetown University Hospital about 6:00 P.M., Nov. 7, 1935, in comatose condition with a tentative diagnosis of acute phenobarbital poisoning.

History.—The patient had received treatment for pulmonary tuberculosis eleven years ago. For the past twenty years she was addicted to phenobarbital and had taken at least gr. lxxv of phenobarbital about thirty-six hours before admission. She shortly lapsed into deep coma. The husband failed to notify the physician immediately, because she had been in a similar state several times before and had recovered. Finally he became alarmed and Dr. Murphy was notified.

Physical Examination.—Comatose. Temperature 99.2° F.; pulse 120, thready; blood pressure 100/60; respiratory rate 24, stertorous. Pupils dilated, equal and regular, reacted to light sluggishly, nystagmus present in both eyes. Corneal reflex present but sluggish. Reflexes in throat absent. Neck flaccid and could be moved easily. Thorax poorly formed, lungs were filled with numerous fine and coarse râles, more pronounced during expiration. Abdominal reflexes absent. All extremities showed a flaccid paralysis; biceps, triceps, and patellar reflexes absent. No voluntary movements present.

Laboratory Findings.—Urine contained albumin and casts. Blood and urine tested for phenobarbital; 580 c.c. of urine obtained by catheterization contained 0.2 mg. of phenobarbital per c.c., or a total of 116 mg. phenobarbital. The blood contained 0.036 mg. of the drug per c.c.

Progress and Treatment.—Besides the usual general supportive measures the patient was treated with picrotoxin.

Table I summarizes the course of treatment and its results.

The data presented in this table show that picrotoxin in a deeply comatose and narcotized patient can be pushed to a considerable extent without toxic manifestations. The total dose of picrotoxin administered intravenously in this patient was 167 mg., and this amount was just sufficient to rouse her from the coma. It also shows that practically each dose of picrotoxin was followed not only by medullary stimulation as manifested by improved respiration and hemodynamic effects but also by distinct awakening phenomena, i.e., by temporary denarcotization of the patient, manifested by return of reflexes, wrinkling the forehead, moving the eyelids, swallowing and attempts to talk. During the course of the continued picrotoxin treatment the patient's blood phenobarbital gradually fell, the fall paralleling the lessening of the narcosis. Since phenobarbital is a drug excreted in the urine only in small percentages, it is not surprising to find that we recovered only 143 mg. of phenobarbital in the urine. It is possible, however, that the patient had voided before admission to the hospital, and thus we lost urine samples containing phenobarbital. In spite of the successful denarcotization of the patient she developed respiratory involvements accompanied by increased temperature (106° F.). She died Nov. 11, 1935 at 12:25 A.M.

Autopsy (by Dr. H. V. Connerty).—Autopsy was performed on Nov. 11, 1935, eleven hours after death.

There was a lung abscess 5 cm. by 3 cm. subjacent to the pleura in the upper lateral portion of the lower lobe of the left lung. Adjacent to this was another abscess 2.5 cm. in diameter. Mucopus could be squeezed out of the small bronchi of this lobe. No definite areas of consolidation could be felt. The upper lobe of the left lung was negative.

There was a thin uniform coating of fibrin over the lower lobe of the right lung. On section an area of consolidation 2.5 cm. in diameter was discovered. Mucopus could be forced out of the bronchi by pressure. The upper and middle lobes of the right lung were negative.

Microscopically the wall of the abscess was composed of necrotic lung tissue infiltrated with fibrin and polymorphonuclear leucocytes. The adjacent alveoli contained an exudate of fibrin in which only a few leucocytes were entrapped. Study of sections taken from other portions of the lower lobe of the left lung and from the lower lobe of the right lung showed essentially identical lesions. There were areas of atelectasis and the lung tissue was markedly congested. The bronchioles were distended by pus in the lumina and in some areas the bronchiolar epithelium had been desquamated. Areas of bronchopneumonia were present. Small miliary abscesses were scattered throughout the pneumonic areas.

TABLE I

THE COURSE OF PICROTOXIN TREATMENT AND ITS RESULTS IN CASE A

DATE	PICROTOXIN MG.	BLOOD PHENOBARBITAL MG. PER C.C.	REACTION OF THE PATIENT
Nov. 7, 1935			
8:30 P.M.	10	0.036	----------
8:58	10	-----	Wrinkles forehead, moves lids, tries to move head, and mumbles
9:18	10	-----	Moves head and eyelids
9:45	7	-----	Same; blood pressure rose from 90/70 to 160/100
10:00	10	-----	Same motor effects
11:30	10	-----	Same motor effects
Nov. 8, 1935			
1:30 A.M.	10	-----	Same
4:30	10	-----	
7:00	10	-----	
9:50	10	0.030	Swallows, opens eyes, raises head, and tries to talk
11:00	10	-----	Moves upper and lower extremities, and head. Speaks unintelligibly
1:00 P.M.	10	-----	Turns on back by own volition
4:00	10	-----	Muscle tone greatly improved; superficial reflexes present. In good condition
7:00	10	-----	Same reaction
9:00	10	-----	Same reaction
Nov. 9, 1935			
1:00	10	-----	Injection followed by spasm lasting for a few seconds. Condition seems improved
10:30	10*	0.020	Patient moves frequently; takes custard and jello which is retained
Nov. 11, 1935		0.014	Patient died†

*Picrotoxin treatment discontinued.

†The postmortem analysis of the organs yielded the following values: liver 0.014, kidney 0.012, skeletal muscle 0.006 and brain 0.015 mg. of phenobarbital per gram of tissue.

The aorta presented lesions of atheroma.

The brain was small and the convolutions were atrophied. The cerebrospinal fluid was increased in amount. The vessels at the base were atheromatous. Microscopically the brain was negative. There was considerable deposition of blood pigment in the liver, kidney, and spleen. The findings in the remaining organs were essentially negative.

This patient died as a result of bronchopneumonia complicated by acute lung abscesses.

B. Mr. F. A., sixty-eight years of age, white. He had taken gr. lviiiss of amytal with suicidal intent on Oct. 25, 1935, about 3:00 P.M. Drs. C. L. Miller and A. J. Connolly were notified and saw the patient at 6:00 P.M.

Physical Examination.—Comatose; pulse 130, thready; blood pressure 70/50; respiration shallow. Pupils constricted, equal, and regular. Corneal reflex sluggish, reflexes in the

throat, abdominal reflexes, superficial reflexes absent. Complete loss of muscle tone, flaccid paralysis of the extremities; no voluntary movements could be elicited.

Progress and Treatment.—This patient was treated with picrotoxin in ampules obtained from Eli Lilly Co. This patient, as the data assembled in Table II show, received a total dose of 24 mg. of picrotoxin. It must be borne in mind that he had taken amytal which is a barbiturate with a shorter duration of action than phenobarbital. This may explain why he required less picrotoxin and reacted more favorably to the antidote than the former case. He also showed clonic twitches and convulsive movements following the administration of later doses of picrotoxin, which subsided after fifteen minutes and were followed by improvement in the muscle tone.

After the patient had received his last intravenous dose of picrotoxin, he soon regained consciousness and recovered completely fifty-eight hours after the administration of the initial dose of picrotoxin. It should be pointed out that this patient did not show any real improvement; in fact, his general condition had become worse until the first intravenous dose of picrotoxin was injected. From this time on his improvement was steady and progressive.

TABLE II

THE COURSE OF PICROTOXIN TREATMENT AND ITS RESULTS IN CASE B

DATE	PICROTOXIN MG.	REACTIONS OF THE PATIENT
Oct. 25, 1935		
6:30 P.M.	3 (muscle)	No effect
9:00 P.M.	3 (muscle)	No effect
Oct. 26, 1935		
9:00 A.M.	3 (muscle)	Respiratory stimulation. Blood pressure gradually rose from 70/50 to 120/80
6:00 P.M.	6 (vein)	Clonic twitches followed by improvement in muscle tone. Moves head and extremities
Oct. 27, 1935		
10:00 A.M.	3 (vein)	Motor response
8:00 P.M.	6 (vein)	Convulsions, movements; marked voluntary attempts to talk. Awakening effect
Oct. 28, 1935		
4:00 A.M.		By this time the patient recovered completely, asked rational questions. Uneventful recovery

COMMENT

These two case reports represent the first clinical attempts to administer picrotoxin in adequate doses in acute barbiturate poisoning. Wood[6] used picrotoxin in 9 cases to denarcotize or resuscitate patients subjected to anesthesia with avertin and volatile anesthetics. He used this drug seven times intravenously and twice subcutaneously, in doses of 3 mg. (in a 10 per cent solution in alcohol or propylene glycol). Only in one case did he repeat the dose and then only once. He gained the impression that the drug has great potency. He himself realized, however, that his doses were inadequate and that further investigation would be necessary to determine the safety of repeating the dose. Arnett[3] used picrotoxin in treating a girl aged three years nine months poisoned with an unknown amount of amytal. He gave two single doses of picrotoxin $\frac{1}{100}$ gr. each. He also used evacuant measures and ephedrine. It is obvious from his description that the patient had not taken a fatal dose of amytal, never lapsed into a real coma, and the use of picrotoxin was probably not indicated.

In our first case of phenobarbital poisoning, picrotoxin was used intravenously in doses of 10 mg. At the end of the treatment a total amount of 167 mg. was administered. During all this period the patient showed only once in the later course of the treatment what might be construed as a toxic manifestation of picrotoxin action. In the other case of amytal poisoning 24 mg. of picrotoxin sufficed to terminate amytal depression. Although the first patient succumbed to respiratory complications so commonly seen in barbiturate poisoning, it represents a rather striking instance of the efficacy of picrotoxin medication. Dougherty's[7] case, reported recently, may serve as a control not receiving picrotoxin medication. Here an acute poisoning from phenobarbital is reported produced by a single overdose. The patient was somewhat younger than ours and was seen first before he passed into a coma. The reflexes were normal, the pulse full and bounding, blood pressure 170/90, respiratory rate 30. Two hours after the patient was seen he passed into a coma and the blood pressure gradually fell to 90/70. The reflexes were normal. Râles were noted over both lobes of the lungs. Within a few hours, the blood pressure rose to 180/90 apparently without any treatment, but the patient was still comatose. The temperature had risen. The blood pressure was 120/80 four hours before death. Death occurred approximately forty-five hours after the drug was taken. It should be noted that this patient was not as severely poisoned with phenobarbital as our first patient, as shown by the presence of reflexes and the relatively high blood pressure. Yet once he became comatose he never came out of this condition and lived only forty-five hours following the ingestion of the poison. Our first patient rallied from the coma following picrotoxin medication, her reflexes returned, she was able to move and eat. And she lived about six days following taking of the drug.

Our second case of amytal poisoning happily also demonstrates the life-saving effect of picrotoxin, whereas both cases show what we call the awakening effect of picrotoxin. In a paper in press we have presented facts proving that picrotoxin has a distinct awakening or cortical effect aside from its medullary stimulant actions which latter are chiefly responsible for stimulating the patients' respiration and circulation.

As indicated by Wood's report[6] the chief concern of the physician in treating barbiturate poisoning will be the manner of administration and posology of picrotoxin. It will be clear to anyone familiar with the principles of rational pharmacotherapeutics that the size of the dose of picrotoxin cannot be accurately fixed. We have shown ([4, 5] and unpublished data) that in animals the dose of picrotoxin sufficient to produce awakening or life-saving effects depends upon the amount of barbituric acid derivatives used in producing narcosis and coma. The deeper the narcosis, the more picrotoxin is required to produce the desired results. The same is also true of human beings. The greater the amount of barbiturate taken by the patient, the deeper the coma and the more picrotoxin will be required to rouse the individual. It is admitted that the physician cannot always tell either the amount of barbiturate taken by the patient or the relative depth of the narcosis. Yet he will have to proceed with picrotoxin medication. How much picrotoxin is he expected to give? The answer as usual is *enough*. We recommend that the

drug be given intravenously because in these cases rapidity of action is an important factor. We also recommend to begin the treatment with a dose of 5 mg. of picrotoxin and wait about twenty or thirty minutes, because if this dose of picrotoxin is effective, it should produce some awakening effect, improvement in muscle tone, circulation and respiration during that interval. Then the dose should be repeated, and if the patient still shows no effects, given again in twenty minutes, but this time 10 mg. may be injected. The first toxic symptoms of picrotoxin are twitches. If these appear, the medication should be discontinued until the patient again becomes lethargic. Incidentally, twitches and convulsions following these moderate doses of picrotoxin indicate that the depression of the central nervous system is diminishing. Then treatment may be resumed but in smaller doses (from 3 to 5 mg.). Picrotoxin treatment should be continued until the patient emerges from the coma.

In one patient 10 mg. of picrotoxin may be sufficient to produce rallying from narcosis and another patient may require 150 or 200 mg. over a period of forty-eight hours to obtain the same result. The treatment requires a continued watching of the patient and cannot be carried out by nonmedical attendants.

Picrotoxin is slightly water soluble, and we recommend using a 0.2 per cent aqueous or physiologic saline solution of which 1 c.c. contains 2 mg. Two and one-half cubic centimeters of this solution injected slowly intravenously should be the initial dose.

We must emphasize that picrotoxin be used only after the diagnosis of barbiturate poisoning has been made beyond a doubt. Koppanyi's macro or micro test[8, 9] should be carried out on the urine or blood of the patient to ascertain the diagnosis, even if the history points to barbiturate poisoning. Owing to the toxicity of picrotoxin in normal individuals or in light narcosis, this drug should be used only in cases when the patient is comatose and possibly flaccid.

It is probable that other aliphatic narcotic poisonings, e.g., with paraldehyde, chloral hydrate, sulphonemethane, avertin, etc., offer indications for picrotoxin medication. We must caution, however, against the use of picrotoxin in cases of opiate poisonings. Kossa[10] has already pointed out that picrotoxin enhances the toxic effects of morphine, and we have shown in unpublished experiments that while picrotoxin counteracts the respiratory and circulatory effects of morphine, it curiously produces neither an awakening nor a life-saving effect in morphine poisoning, in fact, it accelerates a fatal outcome.

The authors take pleasure in acknowledging their indebtedness to Dr. C. L. Miller who has succeeded with Dr. Connolly in treating patient in Case B with picrotoxin. They are also indebted to Dr. B. F. Schaefer who supervised the treatment of patient in Case A, and to Drs. C. R. Linegar and J. M. Dille who performed the phenobarbital determinations on the blood, urine, and organs of Case A.

REFERENCES

1. Maloney, A. H., Fitch, R. H., and Tatum, A. L.: Picrotoxin as an Antidote in Acute Poisoning by the Shorter Acting Barbiturates, J. Pharmacol. & Exper. Therap. **41**: 465, 1931.

2. Maloney, A. H., and Tatum, A. L.: Picrotoxin as an Antidote in Acute Poisoning by the Longer Acting Barbiturates, J. Pharmacol. & Exper. Therap. **44**: 337, 1932.
3. Arnett, J. H.: Ephedrine and Picrotoxin Used Successfully in Amytal Poisoning, J. A. M. A. **100**: 1593, 1933.
4. Linegar, C. R., Dille, J. M., and Koppanyi, T.: The Nature of Barbiturate-Picrotoxin Antagonism, Science **82**: 376, 1935.
5. Linegar, C. R., Dille, J. M., and Koppanyi, T.: Antidotal Action of Picrotoxin in Extreme Cases of Experimental Barbiturate Poisoning, Proc. Soc. Exper. Biol. & Med. **33**: 396, 1935.
6. Wood, P. M.: Denarcotization or Resuscitation of Anesthetized Patients, Anesth. & Analg. **14**: 234, 1935.
7. Dougherty, M. S., Jr.: Toxic Reactions Produced by the Derivatives of Barbituric Acid, South. M. J. **25**: 1145, 1932.
8. Koppanyi, T., Dille, J. M., Murphy, W. S., and Krop, S.: Studies on Barbiturates. II. Contributions to Methods of Barbital Research, J. Am. Pharm. A. **23**: 1074, 1934.
9. Linegar, C. R., Dille, J. M., and Koppanyi, T.: Studies on Barbiturates. XI. Further Contributions to Methods of Barbital Research, J. Am. Pharm. A. **24**: 847, 1935.
10. Kossa, G.: The Biological Effect of Picrotoxin, Hung. Arch. Med. **1**: 469, 1892.

EFFECTS OF COPPER IN THE DIET OF ONE HUNDRED FORTY PERSONS*

J. L. McGhee, Ph.D., Atlanta, Ga.

IN COOPERATION with the employer and 140 employees, the following tests were made on the latter. The number of males and females was about equal and the ages ranged from seven to sixty-two. All lived in one village with the same source of food and water. Two weeks before the tests were begun the subjects were placed on raw, sweet milk with their usual diets, except no blood-regenerating foods were allowed. Then the hemoglobin of each person was determined with the Sahli hemoglobinometer, checked by the Newcomer.

Copper-cobalt-manganese alloy (iron-free) in metallic sheets, coiled, was immersed in each bottle of milk and allowed to remain twenty-four hours. Half of the milk was consumed after twelve hours and the balance after twenty-four hours, supplying 1 mg. copper per day.

Ordinates in the graphs show first the percentage hemoglobin before the copper was added to the diets, then after four and eight weeks. Of the 140

Table I

PERSONS	PER CENT GAIN	PERSONS	PER CENT GAIN
2	0	3	16
8	5	3	17
1	6	3	18
1	7	7	19
1	8	21	20
2	9	1	21
27	10	2	22
2	11	1	24
4	12	3	25
3	13	1	26
8	14		—
36	15	Average gain	14%
		Total 140	

*From the Department of Bio-Chemistry, Emory University.
Received for publication, February 7, 1936.

persons, 138 gave hemoglobin increases from 5 per cent to 26 per cent. No iron was involved except what was in the regular diets. In addition to Fig. 1, Table I shows the numerical data.

The figures following the per cent show the number of persons represented by each graph. Not one of the 140 lost hemoglobin.

Note the composite graph of 14 per cent gain for the 140 tests.

The intake of iron and copper in fresh vegetables during April and May might be responsible for part of the hemoglobin increases; but in another series of similar tests that were made in the fall of the year, the average hemoglobin gain was 13 per cent.

This record should be studied as to the number who failed to appear for the second or third test of the series.

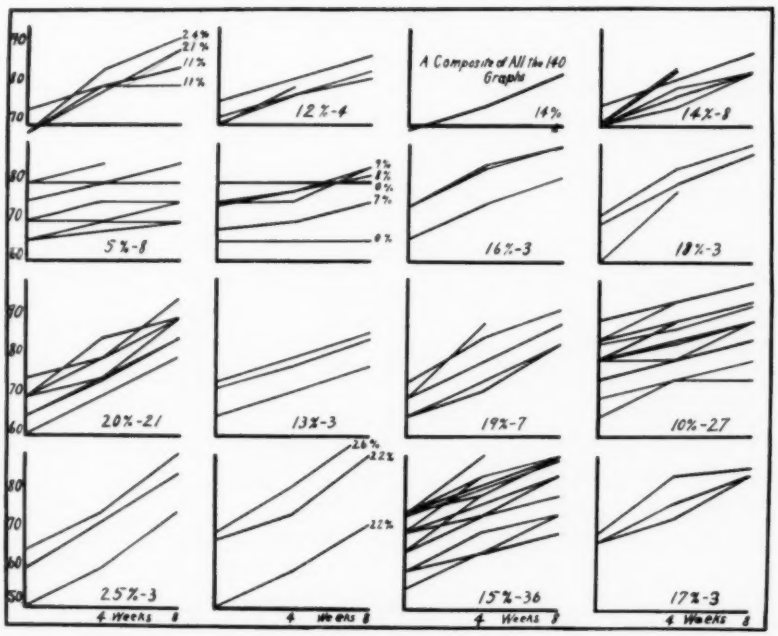

Fig. 1.

Twenty-nine failed to appear for the second blood test.

Seventeen were absent for the third test.

Each of the 140 persons had two or three tests.

Many graphs represent the percentage gain of several persons.

The chief value of the tests consists in the large number of persons involved and the 98.5 per cent of positive response in regeneration of hemoglobin without addition of iron to the diet. Also, in the confirmation of previous reports on the effects of copper in the diet. No pathology was found in any case.

The above work was done by my* method of metallizing foods without the use of salts as such.

*McGhee, J. L.: Science 73: 347, 1931.

BACILLEMIA IN PULMONARY TUBERCULOSIS*

RANDLE C. ROSENBERGER, M.D., AND LOUIS MERVES, B.S., PHILADELPHIA, PA.

IN MEDICINE, it is often the case that a phenomenon is deduced long before any certain demonstration of it has been made, and on the basis of such, prediction treatment is instituted. In science, the validity of either intuitive knowledge or logical compulsion may not be admitted in lieu of controlled experimental demonstration.

Every since 1884, when Weichselbaum described the presence of the "virus of tuberculosis" in the blood of tuberculous patients, much head-scratching, much needle-poking, much eyestrain, and far too much controversy have failed to demonstrate conclusively the presence of a tuberculous bacillemia in the so-called typical case of progressive pulmonary tuberculosis.

Dr. Ernst Löwenstein, of Vienna, claims remarkable results with the use of his method of blood culture. His writings have been prolific, and the number of cases investigated enormous. In a lecture at Escuela de Sanidad, Madrid,[1] he states: "The bacillemia is observed only if the malady is progressive. If the process is only stationary, the culture perhaps (will be) negative." He attempts to explain the failure of other investigators to duplicate his results; on the basis of 14,300 examinations, he gives what he believes to be the principal causes of failure, mainly, insufficient moisture in the media, too short a period of incubation and observation, withdrawal of the blood at an unfavorable stage of the disease, improper sterilization and preparation of the materials and media, and the further fact that only "about 10-15 per cent of the cultures are microscopically positive" when macroscopic results are negative.[3]

Interest in Löwenstein's findings was, as might be expected, widespread. Meersseman and Lumaret[4] examined 95 rheumatic patients and 13 cases of confirmed tuberculosis by Löwenstein's method, and reported one positive culture. Caussimon[5] reported 30 per cent positive results in chronic pulmonary tuberculosis. Popper[6] stated that the extent and duration of the bacillemia depends on the immunity of the patient, and appears only after the activation of a resting tuberculous process or after an acute febrile disease. In an investigation covering 54 cases, Emslie[7] reported 52 negatives, and 2 positives, which when transplanted did not grow out, and produced no lesions when inoculated into guinea pigs. He believed that the two positive cases were due to contaminants. Schwabacher,[8] Weatherall,[9] Cumings,[10] and Pearce[11] all drew similar conclusions. In 150 cases investigated, Busson[12] found 27, or 18 per cent positive cultures; animal tests were positive in 35 cases, 10 where culture was negative, but 2 animal negatives where culture was positive. He

*From the Department of Preventive Medicine and Bacteriology, Jefferson Medical College.
Received for publication, March 26, 1936.

concludes therefrom that the animal method is preferable and that the bacillemia is a sporadic occurrence. Gualdi[13] examined 30 cases and found one positive. Experimentally, Mishulow and Park[14] demonstrated that when rabbits were inoculated intravenously with tubercle bacilli, there occurred first, a rapid fall in the number of bacilli in the blood stream due to localization, followed by a stationary period in which the bacilli occurred in very few numbers, and finally a terminal rise in number.

In an exhaustive monograph, Wilson[15] concludes that "tuberculous bacillemia, except in persons suffering from advanced and grave disease, is a rare and sporadic phenomenon." He states further that the errors present in microscopic positives may be due to contaminants or to artefacts produced by fibrin threads, lipoids, and cellular débris. Siegl[16] published a significant report. Parallel examinations of blood specimens from 140 children were made by Löwenstein and by Maresch separately. Löwenstein found 13.5 per cent positive in the active cases of tuberculosis, 18.9 per cent positive in the inactive cases of tuberculosis, and 23.9 per cent positive in the cases showing no evidence of tuberculosis. Maresch found 5.1 per cent positive in the active cases of tuberculosis, and no positives in the inactive cases, or in the cases showing no evidence of tuberculosis.

In view of the large amount of work done, and the number of cases already studied, it would have been a not very remunerative task to have undertaken the search for a bacillemia in large numbers of patients. In the work done here, we attempted to answer several questions:

1. Will the media used support the growth of small numbers of tubercle bacilli?

2. Are the technics employed efficient in concentrating the organisms if they are present in the blood, and does the technic decrease the viability of the bacilli?

3. Selecting confirmed cases of progressive pulmonary tuberculosis, can the presence of the tubercle bacillus be demonstrated in the circulating blood?

Cohn[18] thinks Löwenstein's medium too complicated and no better than Corper's medium. However, it has been our experience that the Löwenstein media are superior to any of the others.

We used the following medium in the beginning of our series of experiments:

Asparagin	6 gm.
Monopotassium phosphate	4 gm.
Magnesium citrate	1 gm.
Magnesium sulphate	0.4 gm.
Glycerin	60 c.c.
Sterile distilled water	1000 c.c.

The flask containing the above is heated over an open flame until all the ingredients are dissolved. To 250 c.c. of this solution add 6 gm. of potato flour and place in the Arnold for two hours. Cool to 50° C. and add four *whole* eggs and, in addition, one yolk, and 10 c.c. of sterile 2 per cent Congo red solution. Filter through four layers of sterile gauze and tube (about 8 to 12 c.c. in large 16 mm. tubes). Slant in a horizontal autoclave and plug the steam exhaust valve with a cork stopper. The temperature should not rise above 100° C., nor the pressure above 20 pounds. Sterilize for one hour after

the temperature becomes constant. The whole lot of tubes should be incubated for twenty-four hours. If there is no growth, the plugs are then saturated with hot, molten paraffin. There should be some water of condensation at the bottom of each slant. The media must be moist and should be used freshly prepared (fourteen to twenty-one days).

Later, after the receipt of a communication from Dr. Löwenstein, a new recipe was used. Due to the courtesy of Dr. Bucher of the Jefferson Hospital, we were able to use this medium freshly prepared and thus compare it with our own modification.

"Twenty eggs are immersed for one-half hour in a solution of 5 per cent carbonate of soda. Meanwhile a salt solution of asparagin is made:

Asparagin (Merck)	3 gm.
Potassium phosphate (Merck)	1 gm.
Citrate of soda (Kahlbaum)	1 gm.
Magnesium sulphate (Kahlbaum)	1 gm.
Pure glycerin	60 c.c.
Distilled water	1000 c.c.

This solution is sterilized in the Arnold for two hours in flowing steam. It is very stable.

"To 600 c.c. of this solution, one adds 10 c.c. of a 50 per cent solution of potato flour and 60 c.c. of the sterile juice of pressed tomatoes. This mixture is placed for two hours in the water-bath. After cooling to 60° C. one adds 16 eggs and more, the yolks alone of four eggs. Agitate thoroughly, and add 20 c.c. of a 2 per cent solution of Congo red (sterilized for fifteen minutes in the autoclave). When thoroughly mixed, filter through four layers of sterile gauze. Place in sterile tubes. The coagulation is accomplished by heating between 75 and 80° C. in flowing vapor. The tubes are left in the stove and the next day one heats anew for two hours at 75 to 80° C. The tubes are plugged with cellulose. The culture media ought to be prepared at least every two weeks and should be kept in darkness."[2]

The coagulation and final sterilization were carried out by the technic outlined in our method preparation. The only contaminants encountered throughout this series of experiments were in those tubes which had not been incubated before use. Therefore, it is important that the whole batch of tubes be incubated to test for contaminants.

It was found that the first medium was a very good one for growing the tubercle bacillus. After sparse seeding, growth became macroscopic in eight to fourteen days. With proper care, growths may be kept for several months without drying of the medium.

It was found that the second medium was even better (i.e., with the tomato juice); on seedings of approximately equal numbers of organisms, there would be larger colonies on the second medium than on the first, but in every case growth would take place in both media.

It was therefore concluded that if organisms were present and viable, either medium would be efficient in supporting growth.

The technic for the withdrawal of blood was the same as that for ordinary venous puncture, except that the syringes and needles were sterilized in gauze and paper wrappers in the autoclave. The blood was obtained by syringe in 10 c.c. amounts and placed directly into sterile 50 c.c. centrifuge tubes containing 5 c.c. of sterile 10 per cent sodium citrate solution. The blood was then brought immediately to the laboratory and the following technics employed:

Acetic Acid Technic.[17]—"The blood is received in sodium citrate so that it is not coagulated. If coagulation occurs the demonstration is made considerably more difficult. It is then centrifuged strongly and the plasma poured off (this is difficult; we found it better to draw off the plasma with a sterile pipette), the corpuscles are dissolved with 2 per cent sterile acetic acid, again centrifuged, decanted, and then after one washing a few tubes are prepared directly from the sediment. In case the blood taken is not absolutely sterile, the sediment must be mixed with three times the volume of 15 per cent sulphuric acid, centrifuged, washed once with sterile water, decanted, and the tubes inoculated by means of a pipette. If the blood taken was not sterile, or if it contained other bacteria, the first inoculation will show the other bacteria, then after eight to sixty days the first colonies of tubercle bacilli (appear) after the other bacteria in the blood. Often there are many tubercle bacilli in the blood, so that each drop of blood will produce a colony of tubercle bacilli. On the other hand, one may find very isolated colonies. Each tube must be examined microscopically."

Water Technic[2] (as used by us the water was double-distilled in glass, sterilized in the autoclave, and used freshly prepared).

1. Blood specimen centrifuged strongly for thirty minutes.
2. Plasma removed with sterile pipette.
3. Ten to 15 c.c. sterile distilled water added and thoroughly mixed.
4. Centrifuged strongly for thirty minutes.
5. Supernatant liquid poured off, and Steps 3 and 4 repeated until the supernatant liquid became clear, all the blood cells being hemolyzed and the hemoglobin removed, only a small amount of gray débris remaining in the tip of the tube.
6. Fluid decanted and 2 or 3 c.c. of 15 per cent sulphuric acid added and the tube shaken for five minutes.
7. Centrifuged twenty minutes.
8. Washed until all the acid is removed.
9. Distribute sediment into as many tubes as are necessary, reserving enough sediment to examine microscopically.

Of prime importance, it should be seen that the cotton plugs are thoroughly saturated with paraffin.

Controls were made to test for the possible presence of contaminants, and the efficiency of the sulphuric acid treatment. With proper precautions, no contaminants are encountered, but in this series of experiments the sulphuric acid was always included, though inoculations were made in some cases before and after the sulphuric acid treatment of the sediment.

Next, the efficiency of these technics was tested. A *small* loopful of a three-month-old culture was suspended in 10 c.c. of sterile physiologic salt solu-

tion. To 10 c.c. of normal blood was added 0.5 c.c. of this suspension, which was thoroughly distributed by means of a capillary pipette. Two controls were made for each of the blood-lysing technics. In all cases, growth was demonstrable in two weeks. Cohn[18] says in part, that not only is acetic acid toxic to human and bovine tubercle bacilli, but that it is completely bacteriostatic to small numbers while sulphuric acid is not. It should be noted that he used a 6 per cent concentration instead of the 15 per cent used by Löwenstein. Saenz[19] reports that over 20 per cent of sulphuric acid does not affect the viability of human, bovine, or avian types of tubercle bacilli, an observation which coincides with our own experience.

It was therefore concluded that while the acetic acid technic seemed to exercise *some* measure of inhibitive action, yet growth would take place with either method, even though the organism be of a less hardy strain.

We examined specimens of blood from 31 selected cases of progressive pulmonary tuberculosis (each of which was sputum positive, and each of which had cavities demonstrable by x-ray), the acetic acid technic was followed in 20 cases, and the water technic in 11.

CONCLUSIONS

1. The media employed are adequate to support the growth of the bacillus.

2. The revised technic of Löwenstein (employing water to lyse the blood), as used by us, efficiently recovers the organism. The sulphuric acid treatment does not inhibit the growth of the tubercle bacillus.

3. In the 31 cases observed: (a) none showed macroscopic evidence of growth; and (b) two or 6.5 per cent showed *some* microscopic evidence of acid-fast particles which were not capable of producing growth on fresh media.

DISCUSSION

The acetic acid method leaves a comparatively large residue. To properly inoculate this residue 8 or 12 tubes would be required, moreover this tends to lower the concentration of the organisms and increases the numerical chances of failure.

In two cases objects were seen under the microscope which were morphologically like tubercle bacilli, but not brilliantly acid fast. Considerable trouble was experienced at first on this score; objects retained some small measure of the carbolfuchsin, but had a variety of appearance. These were probably due to lipoidal substances present retaining the stain, and threads and débris resembling the morphology of the tubercle bacilli. However, since these did not grow out on transplants, they were considered artefacts.

It would seem therefore that:

a. The bacillemia is not present in pulmonary tuberculosis, or

b. It may be present for a very short time, and then only sporadically following the breakdown of a tubercle into the blood stream. This makes the detection of the tubercle bacillus problematical, or

c. The bacilli may be present in such few numbers that they escaped detection. It is impractical and undesirable to take large amounts of blood

from a patient for this work to increase the numerical chance of demonstrating the bacillemia. It is our belief, however, that if a *single* viable bacillus be implanted on this medium, it will grow.

No positive evidence was presented indicating the presence of a tuberculous bacillemia; if there is a bacillemia, it occurs only rarely and sporadically.

REFERENCES

1. Löwenstein: Foreign Letters, J. A. M. A., p. 2035, 1933.
2. Löwenstein: La méthode d'hemoculture du virus tuberculeux et ses résultats, Ann. Pasteur Inst. **50**: 161, 1933.
3. Löwenstein: Über Fehlerquellen beim kultureltem Nachweis der Tuberkelbazillen im Blut, Deutsche med. Wchnschr. **59**: 1468, 1933.
4. Meersseman and Lumaret: Rhumatisme articulaire aigu et bacillémie tuberculeuse; résultats personnels dans 95 cas étudiés par la méthode de Loewenstein, Presse méd. **42**: 873, 1934.
5. Caussimon: La recherche du bacille de Koch dans le sang circulant des tuberculeux pulmonaires, Presse méd. **39**: 435, 1931.
6. Popper: Die klinische Bedeutung des Tuberkelbazillennachweis im Blut, Deutsche med. Wchnschr. **58**: 89, 1932.
7. Emslie: Detection of Tubercle Bacilli in the Bloodstream in Pulmonary Tuberculosis, Edinburgh M. J. **41**: 141, 1934.
8. Schwabacher: Blood Culture in Tuberculosis, Lancet **1**: 1130, 1931.
9. Weatherall: Tubercle Bacilli in Blood Stream in Human and Animal Tuberculosis, Lancet **1**: 980, 1932.
10. Cumings: Cultivation of Tubercle Bacillus and Occurrence of Tuberculous Bacillemia, Lancet **1**: 983, 1932.
11. Pearce: Detection of Tubercle Bacilli in the Blood, Tubercle **13**: 545, 1932.
12. Busson: Wann finden sich Tuberkelbazillen im Blute und welche Bedeutung hat dies für den Organismus, Med. Klin. **29**: 294, 1933.
13. Gualdi: La bacillemia tubercolare secondo il metodo culturale di Löwenstein, Policlinico **39**: 493, 1932.
14. Mishulow and Park: Tubercle Bacilli in Blood Stream of Rabbits During Course of Infection, J. Prevent. Med. **6**: 95, 1932.
15. Wilson: Tuberculous Bacillemia, Med. Research Council, London, 1934.
16. Siegl: Über den Tuberkelbazillennachweis im Blute von Kindern, Beitr. z. Klin. d. Tuberk. **81**: 556, 1932.
17. Löwenstein: Die klinische Bedeutung der Tuberkelbazillämie, München. med. Wchnschr. **77**: 1662, 1930.
18. Cohn: Analysis of Löwenstein's Method for Isolating Tubercle Bacilli From Blood, J. Infect. Dis. **52**: 214, 1933.
19. Saenz: Etudes sur la bacillémie tuberculeuse; recherches expérimentales et cliniques, Ann. Pasteur Inst. **52**: 645, 1934.

THE RÔLE OF RETICULOCYTES IN MALARIA*

Studies on Benign and Subtertian Malaria

Morris Shushan, M.D., Oscar Blitz, M.D., and C. C. Adams,
New Orleans, La.

NUMEROUS studies on the prophylaxis and therapy of malaria have appeared, but not until recent years have investigators attempted solution of many problems relating to the pathogenesis of the disease.

Fairley and Bromfield,[1, 2] Tareef, Epstein and Gontaewa,[3] and Benajmou[4] apparently established that the reticulocytes during the period of malarial symptoms are below normal levels. They also showed that from six to ten days after treatment is instituted, a maximum rise in reticulocytes occurs, this rise being proportional to the existing anemia. Erythrocyte counts they found varied within wide limits during this period, the average in both benign and subtertian malaria being surprisingly high, 3,900,000 cells per c.mm.

Eaton,[5] while studying multiple malaria-infected erythrocytes, reported a paretic with inoculation tertian malaria in which reticulocytes were frequently infected. We felt that this observation merited further study and believed that investigation of the rôle played by reticulocytes in various types of malaria might be informative. Unfortunately, no cases of quartan malaria have been recently available; our observations are therefore based on cases of benign and subtertian infections.

METHODS OF STUDY

Reticulocyte counts were made according to the method of Haden,[6] our experience convincing us of its superiority. With it, there is possible maximal staining of reticulocytes and because they are free from artifacts, the most minute details of their structure are visible.

Observations were made on seven cases of benign tertian and thirteen cases of subtertian malaria. Sixty-one counts were performed on the subtertian cases, these being equally timed between the paroxysms, care being taken to secure preparations shortly after rises in temperature, or after chills, if present. Thirty-four counts were made on the seven benign tertian cases. With these, effort was made to secure material four to six hours following chills.

A minimum of 2,000 erythrocytes were counted to obtain the reticulocyte percentage. Where discrepancy was noted between the first and second thousand erythrocytes counted, two to four thousand additional cells were counted to insure accuracy. Reticulocytes were expressed in number per cubic millimeter. The parasite counts were established through comparison with the

*Received for publication, March 31, 1936.

reticulocytes, their numbers being also recorded per cubic millimeter. Between 100 and 300 parasites were counted at each observation; in several cases with very few parasites or after treatment, only fifty were counted.

In studying infected reticulocytes, we found two types. In one type, evidence of reticular network was definitely present, the reticular material assuming a rod or club-shaped form; this group was designated "young reticulocytes." In the other type, the reticular material assumed the form of fine, medium or coarse granules which stained definitely blue; these we termed "old reticulocytes." In order to check the accuracy of our color observations and to study Schüffner's granulations in the benign tertian cases, control preparations were made with Wright's stain.

RESULTS

The erythrocytes in benign tertian malaria varied in this series between 2,870,000 per c.mm. and 4,510,000 per c.mm., averaging 3,892,000 per c.mm. The erythrocyte counts in subtertian malaria varied between 1,760,000 and 5,550,000, the average being 4,065,000 per c.mm. These averages compare favorably with those reported by Fairley and Bromfield,[1] who obtained averages of 3,990,000 and 3,926,000 in benign and subtertian, respectively.

The results obtained in seven cases of benign tertian malaria are tabulated in Table I. Reticulocytes per c.mm. varied between 15,900 and 106,590, the

TABLE I

STUDIES OF BENIGN TERTIAN MALARIA

CASE	HOURS AFTER CHILL	RETICULOCYTES	PARASITES TOTAL	INFECTED RETICULOCYTES (YOUNG)	INFECTED RETICULOCYTES (OLD)	INFECTED ERYTHROCYTES (MATURE)	RETICULOCYTES TO ERYTHROCYTES	INFECTED TO NON-INFECTED RETICULOCYTES	INFECTED RETICULOCYTES TO INFECTED MATURE ERYTHROCYTES
1	5	82,300	12,943	3,234	2,333	5,565	1: 47.6	1:13.0	1: 1.0
	8	54,600	19,430	7,095	4,080	7,772	1: 71.4	1: 3.8	1: 0.7
2	½	94,050	27,100	11,470	135	12,420	1: 44.0	1: 7.1	1: 1.0
	6½	106,590	26,092	2,818	8,349	12,524	1: 38.0	1: 8.5	1: 1.1
3	5	22,550	26,725	3,173	5,597	15,335	1:200.0	1: 5.0	1: 1.7
	12	24,800	12,300	0	369	11,316	1:182.0	1:67.0	1:30.7
4	6½	24,400	21,000	3,102	2,741	12,300	1:166.0	1: 3.2	1: 2.1
5	4	30,170	12,068	1,207	2,172	8,086	1:142.0	1: 7.9	1: 2.4
6	5	15,900	6,510	607	955	4,037	1:200.0	1: 9.0	1: 2.6
	3	19,000	15,800	1,896	0	11,376	1:166.0	1: 9.0	1: 6.0
	10	21,200	10,440	1,044	0	7,308	1:143.0	1: 9.0	1: 7.0
7	6	90,400	2,260	271	0	1,628	1: 31.0	1:332	1: 6.0

average count being 48,830 per c.mm. Cases 1 and 3 showed definite rises in reticulocytes during and shortly after chills. This phenomenon was not observed in the remaining cases. No regular reticulocyte rhythm could be demonstrated although irregular fluctuations were noted in all cases over the entire period of observation.

The total number of parasites per c.mm. varied between 2,260 and 27,100, the average being 16,055 per c.mm. Parasite counts in the individual cases of tertian malaria varied little during the cycle of infection. The number of

infected young reticulocytes varied between 271 and 11,470 per c.mm., excluding a twelve-hour observation on Case 3, in which no young infected reticulocytes were noted. The proportion of infected young reticulocytes varied inversely with the number of hours after the chill. Few infected reticulocytes showed well-developed reticular networks; uninfected reticulocytes, however, revealed well-developed reticulations occupying more than half of the cell. The infected "young reticulocytes" possessed, as a rule, scanty and disorganized reticular skeins. The infected "old reticulocytes" varied between none, as in Cases 6 and 7, and 8,349 per c.mm. as in Case 2. The general average was 2,227 per c.mm. As a rule, the older the parasite, the higher was the percentage of infected "old reticulocytes." The infected mature erythrocytes varied between 1,628 and 15,335 per c.mm., the average being 9,139 per c.mm. These figures include only infections with parasites in the ring stage.

The ratio of reticulocytes to erythrocytes in benign tertian malaria varied between 1:31 and 1:200, the average being 1:80.

The ratio of infected to noninfected reticulocytes averaged 1:8.3. Case 7 presented a ratio of 1:332, but in this instance the parasite count itself was extremely low, being only 2,260 per c.mm. The maximal ratio observed was 1:3.2 in Case 4 where the parasite and the reticulocyte counts were almost equal.

The ratio which best expresses the susceptibility of reticulocytes to infection is that of infected reticulocytes (both young and old forms) to infected mature erythrocytes. This varied from 1:0.7 in Case 1, eight hours after the chill, to 1:30.7 in Case 3 in a preparation made twelve hours after the chill. In Case 6, a paretic with inoculation malaria undergoing transition of cycles, rather higher ratios were found. In Case 7 with a very low parasite count, a high ratio was also found. The general average calculated from the average number of infected reticulocytes and infected mature erythrocytes in the ring stage was found to be 1:1.8.

Thirteen cases of subtertian malaria were studied, as seen in Table II. In these, the reticulocytes varied between 8,400 and 488,310 per c.mm., their average being 62,185 per c.mm. Case 6 presented a very high reticulocyte count resulting from a six weeks' infection, with an erythrocyte count of only 1,760,000. As in the cases of benign tertian, although marked variations in reticulocyte levels were observed during the cycle, the reticulocyte peaks showed no regularity as to their time of occurrence, and their levels could not be correlated in any way with the malarial paroxysms.

The parasite counts per cubic millimeter varied between none, observed in Cases 8 and 9 of this series, and a maximum of 280,000 per c.mm., the average being 35,974 per c.mm. The counts on individual cases revealed very wide fluctuations, seemingly not dependent on the time the preparations were made.

In every case except one in this series, certain preparations failed to reveal infected reticulocytes. It is probably true that a small number of infected reticulocytes might be found on any preparation if each were examined ex-

haustively. The maximal count of infected reticulocytes was 4,200 per c.mm., the average was 297 per c.mm. The majority of infected reticulocytes observed, unlike those with benign tertian, presented very well-preserved networks. It was also noted that no infected reticulocytes were demonstrated on preparations presenting extremely low parasite counts.

No infected mature erythrocytes could be demonstrated on two preparations: Cases 8 and 9. The maximum observed was 280,000 per c.mm. and the average 35,478 per c.mm.

The ratio of reticulocytes to erythrocytes varied between 1:4 and 1:435, the average being 1:65. The maximal ratio of infected to noninfected reticulocytes was 1:7, the average being 1:207. Ratios naturally could not be calculated in those cases where no infected reticulocytes were found.

Rather high ratios were observed in Case 3 of this series, in which the parasite count was very high and the reticulocyte count low. Again, where the situation was reversed, as in Case 6, the reticulocyte count being high and

TABLE II

STUDIES OF SUBTERTIAN MALARIA

NO.	RETICULOCYTES PER C.MM.	PARASITES PER C.MM.	INFECTED RETICULOCYTES PER C.MM.	INFECTED MATURE ERYTHROCYTES	RETICULOCYTES TO ERYTHROCYTES	INFECTED TO NONINFECTED RETICULOCYTES	INFECTED RETICULOCYTES TO INFECTED MATURE ERYTHROCYTES	
1	120,100	63,400	1,300	62,770	1: 20	1: 65	1: 32	Max.
	86,100	1,600	0	7,076	1: 28	–	–	Min.
	106,100	31,856	595	31,250	1: 26	1:178	1: 52	Av.
2	43,000	88,050	1,150	87,980	1: 95	1: 46	1: 24	Max.
	20,340	5,850	0	5,780	1:200	–	–	Min.
	29,270	41,728	475	41,253	1:151	1: 60	1: 86	Av.
3	33,500	280,000	4,200	280,000	1:114	1: 7	1: 26	Max.
	8,400	16,600	0	16,250	1:435	–	–	Min.
	18,670	83,053	923	82,130	1:214	1: 17	1: 89	Av.
4	43,520	116,900	1,160	114,730	1:125	1: 40	1:100	Max.
	21,760	3,380	0	3,380	1:250	–	–	Min.
	32,782	60,030	415	59,600	1:162	1: 78	1:144	Av.
5	50,250	37,700	377	37,500	1:100	1: 81	1: 99	Max.
	27,775	2,260	0	2,260	1:200	–	–	Min.
	39,560	27,700	94	27,600	1:141	1:420	1:293	Av.
6	488,310	14,713	1,315	10,728	1: 4	1:253	1: 4.7	Max.
	303,500	3,870	0	3,370	1: 7	–	–	Min.
	346,045	8,724	624	6,040	1: 5.7	1:553	1: 9.6	Av.
7	31,780	11,350	0	11,350	1:200	–	–	Max.
	22,700	4,540	0	4,540	1:143	–	–	Min.
	29,060	8,320	0	8,320	1:155	–	–	Av.
8	54,600	17,550	0	17,550	1: 71	–	–	Max.
	21,450	0	0	0	1:222	–	–	Min.
	36,320	6,350	0	6,350	1:107	–	–	Av.
9	45,675	5,337	0	5,337	1: 95	–	–	Max.
	17,000	0	0	0	1:250	–	–	Min.
	24,368	1,605	0	1,605	1:177	–	–	Av.
10	35,550	30,000	30	29,970	1:111	1: 99	1:993	Max.
	29,800	19,200	0	19,200	1:133	–	–	Min.
	32,675	24,600	15	24,585	1:121	1:2197	1:1639	Av.
11	34,160	46,970	465	46,505	1:125	1: 36	1:100	Max.
	17,080	32,025	0	32,025	1:250	–	–	Min.
	25,620	39,498	233	39,265	1:167	1:109	1:168	Av.
12	70,180	19,500	487	18,526	1: 45	1:141	1: 40	
13	17,765	114,700	0	114,700	1:285	–	–	

the parasite count low, we note that the ratio of infected to noninfected reticulocytes is small. The maximal ratio of infected reticulocytes to infected mature erythrocytes was 1:4.7 in Case 6, the average being 1:119.

A comparison of the general averages in the two types of malaria, as shown in Table III, reveals quite distinct differences. The total parasite count averages 16,055 per c.mm. in benign tertian against 35,974 in subtertian malaria. We feel that it is a well-recognized observation that subtertian infections will produce a greater number of parasites in the peripheral blood stream. The total parasite count in our benign tertian cases would have been even lower were it not for the fact that we excluded four cases which showed very few parasites, two of which had spontaneous remissions.

TABLE III

AVERAGES

TYPE	ERYTHROCYTES	RETICULOCYTES	PARASITES TOTAL	INFECTED RETICULOCYTES (YOUNG)	INFECTED RETICULOCYTES (OLD)	INFECTED ERYTHROCYTES MATURE)	RETICULOCYTES TO ERYTHROCYTES	INFECTED TO NONINFECTED RETICULOCYTES	INFECTED RETICULOCYTES TO INFECTED MATURE ERYTHROCYTES
Benign tertian	3,892,000	48,830	16,055	2,993	2,227	9,139	1:80	1: 8.3	1: 1.8
Subtertian	4,065,000	62,185	35,974	297	0	35,478	1:65	1:207	1:119

The number of infected reticulocytes is found to differ markedly in the two types of malaria. In benign tertian, the average number of infected reticulocytes was found to be 5,220 against 297 per c.mm. in the subtertian. That infected reticulocytes are present in large numbers in the benign tertian type is also revealed by the ratio or proportion of infected reticulocytes to infected mature erythrocytes in the ring stage. In tertian malaria this ratio averages 1:1.8; in subtertian this ratio is 1:119.

The ratio of infected to noninfected reticulocytes in the two types of malaria varies considerably. In benign tertian our observations showed one infected to 8.3 noninfected reticulocytes. In the subtertian variety, we found a ratio of 1:207. The reticulocyte-erythrocyte ratio in benign tertian malaria was found to be 1:80, while the infected reticulocyte to infected mature red cell ratio was 1:1.8. Here it will be seen that infected reticulocytes exist in much greater numbers than would be expected from the percentage of immature cells present in the peripheral circulation. A comparison with the subtertian cases reveals a reticulocyte-erythrocyte ratio of 1:65. The infected reticulocyte to infected mature erythrocyte ratio is 1:119. Infected reticulocytes exist in smaller numbers than would be expected from the reticulocyte percentages.

DISCUSSION

These data lead us to believe that merozoites selectively infect reticulocytes in benign tertian malaria, or that reticulocytes are more susceptible to infection than mature erythrocytes. Merozoites of benign tertian type may

grow and develop in mature erythrocytes but infection of immature cells is of great importance. In the subtertian type, it appears probable that the numbers of immature cells infected by merozoites will depend upon the number of reticulocytes available at that particular time. These parasites apparently grow with equal facility in mature or immature erythrocytes.

The striking difference in the behavior of these two varieties of malaria may logically explain the differences in the parasite counts. Subtertian malaria produces a heavier infection associated with a higher parasite count in the peripheral circulation because of the fact that subtertian merozoites infect erythrocytes of any type. Under the circumstances optimal conditions exist for the propagation of the infection and the continued abundant growth of the parasites. In the benign tertian variety, we feel that the lower parasite counts are in large measure due to the fact that opportunity for the merozoites to enter and infect erythrocytes is limited by their obvious preference for immature red cells. We must also bear in mind that reticulocytes comprise only approximately 1 per cent of all available erythrocytes, and that reticulocytes, during the period of malarial symptoms, do not exceed normal figures. Even though the benign tertian parasite destroys an appreciable number of reticulocytes, factors seem to operate which prevent a rise in the count. Oddly, enough, reticulocytes average distinctly higher in the subtertian cases.

Further, we may apply some of our newer knowledge of reticulocytes to this malarial problem. The severity of the clinical manifestations of subtertian malaria are not due only to the high parasite count. Recently, investigators[7-9] have called attention to the fact that reticulocytes are not carriers of oxygen; in fact, they require oxygen in order that they may exist. Further, they have been unable to demonstrate maturation of reticulocytes in the circulation after their discharge from the bone marrow. As a response to therapy in anemias, the reticulocyte crisis which is observed is really an unavoidable evil; and delivery of nonfunctioning erythrocytes precedes a gradual rise in erythrocytes due to the liberation of functioning mature cells from the bone marrow. If these observations are true, then we can readily see why a destruction of reticulocytes by smaller numbers of benign tertian parasites will produce less severe manifestations than subtertian infections where large numbers of parasites operate to destroy functioning red cells.

The idea has been expressed by Eaton[5] that the sudden reduction in the number of available reticulocytes at the time of the chill is one factor responsible for spontaneous cure in malaria. We have had the opportunity to study two cases of benign tertian infections in which spontaneous remission of symptoms occurred. In one of these cases, a striking diminution of reticulocytes was noted at the time the chill was expected to occur. In another case, remission of symptoms occurred with no definite change in the reticulocyte level. This question, we believe, must remain subjudice at present.

Reticulated erythrocytes appear in a Wright's stained preparation either in the form of polychromatophilic red cells or as erythrocytes presenting basophilic stippling.[10] As Schüffner's granulations definitely characterize benign malaria, and as reticulocytes are selectively infected by the tertian parasite, it is possible that these granulations may result from degenerative changes

induced in reticulocytes by the growth of the tertian parasite. A comparative study of preparations stained by Wright's alone and by brilliant cresyl blue counterstained by Wright's has shown that in certain instances, one type of staining reveals a predominance of Schüffner's granulations in the infected erythrocytes while preparations stained by brilliant cresyl blue show a majority of erythrocytes with evidence of basophilic reticular material.

SUMMARY AND CONCLUSIONS

A study was made of seven cases of benign and thirteen of subtertian malaria. Ninety-five preparations were examined and form the basis for this communication. An attempt was made to secure counts on benign tertian cases six hours or less after the chill. Subtertian preparations were made at various times during the cycle. Erythrocyte, reticulocyte, total and differential parasite counts were determined.

Results to date show that:

1. Reticulocyte counts during period of malarial symptoms are normal or subnormal, excluding cases of severe anemia.

2. No regular reticulocyte rhythm could be demonstrated, although irregular fluctuations were noted.

3. In cases of malaria presenting only small rings, in which a definite diagnosis cannot be made between benign and subtertian infections, reticulocyte preparations may be studied to aid in the separation of the two types of malaria.

4. Reticulocytes are selectively infected by the tertian parasite, whereas in subtertian malaria, reticulocytes and mature erythrocytes are infected with equal facility. This may explain why subtertian malaria is associated with a higher parasite count in the peripheral circulation than in benign tertian malaria.

5. Schüffner's granules may be related to the basophilic stippling of reticulocytes.

REFERENCES

1. Fairley, N. H., and Bromfield, R. J.: Laboratory Studies in Malaria and Blackwater Fever, Tr. Roy. Soc. Trop. Med. & Hyg. **27**: 289, 1933.
2. Fairley, N. H.: Studies on Blood Regeneration in Tropical Diseases, Tr. Roy. Soc. Trop. Med. & Hyg. **27**: 545, 1934.
3. Tareef, Epstein, and Gontaewa: Quoted by Fairley and Bromfield.[1]
4. Benajmou: Quoted by Fairley and Bromfield.[1]
5. Eaton, P.: Susceptibility of Red Cells to Malaria, Am. J. Trop. Med. **14**: 431, 1934.
6. Haden, R. L.: Technic of a Blood Examination, J. Lab. & Clin. Med. **17**: 843, 1932.
7. Mermod, C., and Dock, W.: Fragility and Maturation of Reticulocytes, Arch. Int. Med. **55**: 52, 1935.
8. Osgood, E. E., and Wilhelm, M. M.: Reticulocytes, J. Lab. & Clin. Med. **19**: 1129, 1934.
9. Murphy, W. P.: Production of Reticulocytes, Erythrocytes and Hemoglobin in Anemia, Arch. Int. Med. **52**: 829, 1933.
10. Whitby, L. E. H., and Britton, C. J. C.: Relation of Stippled Cell and Polychromatic Cell to Reticulocyte, Lancet **1**: 1173, 1933.

ON THE NATURE OF THE ANTIPERNICIOUS ANEMIA PRINCIPLE I.*

HENRY R. JACOBS, M.D., CHICAGO, ILL.

HITHERTO the chemical identity of the essential substance in therapeutically active antipernicious anemia preparations has not been disclosed. Common experience has shown that activity is lost when the material is subjected to drastic purification and crystallization procedures. Because of this difficulty attempts were made to synthesize substances resembling those in liver extract without first learning the chemical structure of the active principle. There are several considerations which are of aid in this undertaking.

1. That normal gastric activity elaborates the active principle is now certain,[1] but whether failure of gastric function in pernicious anemia is the cause or the result of the disease is not known.

2. That the active principle is destroyed in the gut is indicated by the definitely lessened effectiveness of material given by mouth compared with that given parenterally, and also by the hemic changes in sprue and in fish tapeworm disease.

3. The cause of spontaneous remissions in untreated cases of pernicious anemia has not been explained. Since recovery of gastric function is improbable, a temporary partial failure of tryptic digestion may be responsible.

4. If the active principle were solely of the nature of an internal secretion[1] of the stomach, it would probably be absorbed by the blood where secreted. On the contrary, it is found inside the stomach and gut, and in all likelihood discharges its function there. Perhaps this function is to modify the course of digestion of normal foods (tryptic digestion?).

5. The author has found a definite difference between normal and pernicious anemic, fasting, gastric content in the reaction with alkaline picric acid solution. Whereas normal filtered fasting gastric content, or a phosphotungstic acid precipitate of it, will redden alkaline picric acid solution after ten or fifteen minutes' heating in the water-bath, that from subjects with pernicious anemia will not do so.† This difference does not depend on simple reducing power, because neither the normal nor the anemic gastric content will change a sensitive oxidation-reduction indicator (2,6-dichlorophenol-indophenol). (The alkaline picric acid reaction is thought not to depend on simple reduction.[2])

6. Abderhalden[3] found that peptic digestion of meat increased and that tryptic digestion of meat decreased the strength of the picric acid reaction. He considers[4] a positive reaction to indicate the presence of proteins, proteoses, pep-

*From the Department of Medicine, University of Chicago.
Received for publication, March 17, 1936.

†Mix 1 c.c. of saturated picric acid solution with 6-8 c.c. of saturated sodium carbonate solution. Add 1 c.c. of filtered fasting gastric content, or a phosphotungstic acid precipitate of it. Heat in a boiling water-bath for 10-20 minutes.

tones and diketopiperazines (and N-N′ substituted diketopiperazines) and a negative reaction to indicate that only peptides, amino acids and O-O′ substituted diketopiperazines may be present in protein hydrolysates.

Uwatoko and others[5] have noted that pepsin-HCl and erepsin do not affect diketopiperazines but that trypsin and trypsin plus kinase attack them vigorously.

7. The tissues primarily involved in pernicious anemia abound in glycoproteins. Glycoproteins are constituted in part of hexoseamine. Glucosamine and mucin both give a positive picric acid reaction.

8. All purified active antipernicious anemia preparations tested gave a positive picric acid reaction. Clinical activity seems to be related to the power of changing hemoglobin to met-hemoglobin.[6] This apparent capacity for both oxidations and reduction suggests aldehydic behavior, or better perhaps, the presence of active carbonyl groups.

9. The reddish color of active liver preparations, and the tendency of this color to deepen on warming, suggest the presence of a substance which will polymerize readily to form deep red compounds. Aldehyde is such a substance.

10. The hydrolysates of mucins and mucoids uniformly contain acetic acid. This acetic acid is thought to come from the acetyl group attached to the nitrogen of the hexoseamine in the glycoprotein.

11. Trypsin is known to be actively inhibited by aldehyde. These considerations suggested several possibilities:

(a) That therapeutic activity may depend on an oxidation-reduction phenomenon. (b) That glucosamine or one of its derivatives might be the active substance. (c) That the nitrogen of the amino-compound might bear an acetyl or other two-carbon chain which will separate readily as aldehyde or acetic acid. (d) That the active substance might be anhydride in nature, and composed of several different groups. Leucine and tyrosine are associated with hexoseamine in mucoids, and they have been found in the study of active material.[7] (e) That the active substance might be necessary for the formation of normal glycoproteins and glycolipids. On the basis of these considerations several preliminary experiments were performed:

1. It was found that the percentage of reticulocytes in grain-fed pigeons[8] would rise after intramuscular injection of 1 or 2 c.c. of 10 per cent acetaldehyde in water. But since it is known that these animals respond well to meat alone, this response may possibly follow from resorption of muscle damaged by the injected acetaldehyde.

A patient with untreated pernicious anemia was given small doses of 10 per cent acetaldehyde intramuscularly (5 c.c. in five days). Although the general symptoms and the appetite improved, the reticulocyte response was equivocal (not over 2 per cent) and the blood picture showed no improvement.

2. Two untreated cases of pernicious anemia showed no reticulocytosis after subcutaneous injection of glucosamine. One showed no reticulocytosis after subcutaneous injection of diacetyl-glucosamine. Both substances were given in substantial dosages. One case of pernicious anemia which had not responded to ventriculin by mouth showed a reticulocytosis of 14 per cent five

days after subcutaneous injection of a water solution of the product of interaction between acetaldehyde and glucosamine. Three other previously untreated cases failed to respond to similar treatment.

3. In methyl alcohol acetaldehyde reacts with glucosamine, glycine, aminobutyric acid, aminovaleric acid, valine and aminocaproic acid and ammonia to form red substances resembling in color and solubilities the active liver extract of commerce. These substances are precipitated by phosphotungstic acid. Glucosamine standing alone or heated in glycerin forms a similar substance. These substances seem refractory to purification and crystallization procedures, but seem to resemble the "animal gums" and aldehyde resins in general behavior. Since aspartic acid, glutamic acid and glycine anhydride do not react in this manner, it may be concluded that the basicity of the amino group may be related to the reaction, and they further resemble liver extract in forming a reddish solution with a green fluorescence when warmed with 70 per cent sulphuric acid containing a little betanaphthol. However, the latter reaction is imitated by both acetaldehyde and paraldehyde, as well as by glucosamine itself.

It is believed that the reddish color of active liver extracts may be that of a polymer of aldehyde attached to the active principle. Whether it is concerned with therapeutic activity, and whether it is united to the nitrogen of amino-compounds capable of forming (anhydride?) complexes remains for further study. Glucosamine and the common amino acids must be examined in this regard, and leucine and tyrosine deserve especial attention. A negative Millon reaction does not exclude tyrosine.[9] And serine, for example, uniting with acetaldehyde with the elimination of water, yields $C_5H_9NO_3$, which is also the formula for oxyproline and resembles that of the bios of Eddy, Kerr, and Williams[10] derived from autolyzed yeast. Monoacetyl-serine and glutamic acid have the same empirical formula, $C_5H_9NO_4$. Of course, there are many other possibilities as well.

The alkaline picric acid reaction may yield important information about the difference between the secretions of the gastrointestinal tract of normal and anemic subjects.

RÉSUMÉ

Preliminary studies indicate that there is a difference between normal and pernicious anemic subjects in the reaction of their fasting gastric contents toward alkaline picric acid solution.

The possible structure of the active principle in antipernicious anemia substances is briefly discussed.

One case of pernicious anemia which had not responded to ventriculin by mouth exhibited a reticulocytosis of 14 per cent five days after subcutaneous injection of a water solution of the product of interaction between glucosamine and acetaldehyde.

REFERENCES

1. *See* Greenspon, E. A.: J. A. M. A. **106**: 266, 1936.
2. Saski, Takaoki: Biochem. Ztschr. **114**: 63, 1921.
Abderhalden, Emil, and Komm, Ernst: Ztschr. f. physiol. Chem. **139**: 181, 1924.
Dox, Arthur W.: Ztschr. f. physiol. Chem. **150**: 118, 1925.

3. Abderhalden, Emil, and Komm, Ernst: Ztschr. f. physiol. Chem. **140**: 99, 1924.
4. Idem, and in many other papers by Abderhalden and others.
5. Uwatoko, Y.: Ztschr. f. physiol. Chem. **139**: 76, 1924.
Matsui, Jujiro: J. Biochem. (Japan) **17**: 253, 1933.
Ishiyama, Takashi: J. Biochem. (Japan) **17**: 285, 1933.
6. Duesberg, Richard, and Koll, Werner: Arch. f. exper. Path. u. Pharmakol. **162**: 296, 1931.
Felix, K., and Frühwein, H.: Ztschr. f. physiol. Chem. **216**: 173, 1933.
7. Cohn, E. J., McMeekin, T. L., and Minot, G. R. : J. Biol. Chem. **87**: xlix, 1930.
Vaughan, Janet, Muller, Gulli Lindh, and Zetzel, Louis: Brit. J. Exper. Path. **11**: 456, 1930.
Felix, K., and Frühwein, H.: Ztschr. f. physiol. Chem. **216**: 173, 1933.
8. Vaughan, Muller, and Zetzel: See reference 7.
9. Abderhalden, Emil, and Stix, Walter: Ztschr. f. physiol. Chem. **129**: 143, 1923.
10. Eddy, Walter H., Kerr, Ralph W., and Williams, R. R.: J. Am. Chem. Soc. **46**: 2846, 1924.

PANCREATIC EXTRACT (TISSUE EXTRACT NO. 568) XIV*

Its Use in the Treatment of Hypertension

Joseph B. Wolffe, M.D., and Victor A. Digilio, M.D., Philadelphia, Pa.

A TRANSITORY decrease in animal blood pressure following the intravenous administration of insulin-free pancreatic extracts has been described.[1-6] Successes following the intramuscular administration of such an extract in lowering the blood pressure of individuals suffering from hypertension have been reported.[4, 12-19] Comparatively successful results in relieving some of the symptoms frequently seen in hypertensives, particularly intermittent claudication, angina pectoris and headache, have also been recorded.[1, 2, 6-9, 11] This led to a study of the effect of deinsulinized† pancreatic extract (tissue extract No. 568) in a series of 150 unselected cases of hypertension.

Three groups of 50 patients each were established at random, varying in ages between twenty-three and seventy-one years. The patients in Group 1, serving as controls, received no medication. Those in Group 2 were given intramuscular injections of sterile normal saline solution, and those in Group 3 were given intramuscular injections of tissue extract No. 568. The bed-resting patients of Groups 2 and 3 received from 24 to 48 daily injections.

After a prolonged period of observation to detect any possible results from the injections, half of the patients in Groups 1 and 2 were given 24 or more injections of tissue extract No. 568. At the same time twenty-five of the patients in group 3 were transferred to the control Group 1. This was done to obtain added controls and to rule out possible psychic factors.

To eliminate possible variations due to individual errors, the same observer made blood pressure determinations with each patient in the recumbent position, taking care that the patient's arm and the apparatus were on the same

*From the Department of Medicine, Temple University Medical School.
Received for publication, March 24, 1936.
†In some of the tissue extract No. 568 used clinically, insignificant traces of insulin were present. This will be discussed in a subsequent paper in this series.

level as the heart. Patients in all groups were allowed to rest for one hour before measuring their blood pressures. Blood pressure readings on the bed patients were taken three times daily; on ambulatory patients, twice weekly. No untoward effects were observed following the administration of approximately 3,800 injections of tissue extract No. 568 in this investigation, with the exception of slight pain at the site of injection; mild urticaria in 2 patients, a sterile area of fat necrosis in 1 and a staphylococcic abscess in a diabetic patient.

Daily and diurnal variations of 50 to 80 mm. in the systolic and of 10 to 20 mm. in the diastolic pressures were observed in the bed-resting and in the ambulatory patients receiving tissue extract therapy. Variations in the control group ranged between 20 and 40 mm. in systolic and between 5 and 10 mm. in diastolic pressures. Consistently higher blood pressures were observed in the ambulatory group, which showed a moderate fall in blood pressure when resting in bed. The injection of tissue extract caused but slight lowering of the average blood pressure of the treated group as compared with that of the control group.

In 70 per cent of the patients of the treated group (receiving 24 or more injections), there was a slight and definite, though transitory, lowering of the blood pressure. Two patients of the control group showed an average lowering of 70 mm. in the systolic pressure, but the diastolic remained stationary and signs of cardiac failure appeared. Eight of the treated cases showed an average diminution of 80 mm. in the systolic blood pressure and 30 mm. in the diastolic for an average duration of one year without developing any of the signs of cardiac failure. These 8 patients were all of the simple hypertensive types, varying in age from forty-eight to sixty-seven years, and showing comparatively normal renal function. None of the cases of essential hypertension showed any lasting significant decrease in blood pressure. Sixty-two per cent of our treated patients obtained relief from either headache, dizziness, angina pectoris, or intermittent claudication. This was seen most frequently following 3 to 4 injections in some, 8 to 12 injections in others. There was no parallelism between the symptomatic relief and the lowering of blood pressure. We believe relief is associated with metabolic changes, probably as a result of improved lipoid metabolism.

In reviewing the cases of hypertension, whose blood pressures were lowered following the administration of tissue extract No. 568, as compared with the very large number in whom the blood pressure was not materially affected, we could not ascertain any significant difference in the history or physical make-up which could aid us in recognizing cases suitable for this type of therapy.

Doctors Hekimian and Roberts of the University of Buffalo Medical School collaborated in the studies of "essential hypertension." Detailed and controlled studies were made by them on 8 patients, aged between eighteen and forty years, who did not show any eyeground or renal changes. They were injected with tissue extract No. 568 in doses between 1 c.c. and 10 c.c. daily for weeks. No effect was observed on the blood pressure of 2 patients; the other 6 showed decreases up to 40 mm. within a few hours. If the injections

were repeated at frequent intervals, the blood pressures remained at a lower level; if the injections were discontinued for a week or longer, the blood pressures showed a definite tendency to return to the original levels.

Cerebral vascular accidents occurred in 5 of our 150 cases, 4 in the control group and 1 in the treated group. One of the control group developed coronary thrombosis with acute insanity. One of each group developed hypertensive psychoses, which differed in their symptomatology, course and response to therapy from the acute psychosis following coronary thrombosis. These cases will be reported in detail elsewhere. Three patients in the control group and one of the treated group died during the period of observation.

CONCLUSIONS

1. In 8 of 108 patients suffering from hypertensive cardiovascular disease, a striking lowering in the systolic and diastolic blood pressure was observed following the administration of tissue extract No. 568. Sixty-two per cent of the treated patients obtained symptomatic relief which lasted for over a year.

2. In the remaining patients treated with this extract, the effects on the blood pressure were similar to those in the control group.

3. In cases of essential hypertension a fairly constant, slight, temporary lowering of blood pressure was observed which lasted only while tissue extract was being administered.

4. Symptomatic relief was obtained in many patients whose blood pressures remained at constant, original, high levels.

We wish to express our appreciation to Sharp and Dohme for the liberal supply of tissue extract No. 568 used in this investigation.

REFERENCES

1. Wolffe, J. B.: The Therapy of Tissue Extract (''Desympatone''), Trans. Am. Therap. Soc., p. 31, 1931.
2. Wolffe, J. B., Findlay, D., and Dessen, E.: Treatment of Angina Pectoris With a Tissue Vasodilator Extract, Ann. Int. Med. **5**: 625, 1931.
3. Munch, J. C., and Wolffe, J. B.: The Effect of Norit on the Depressor Constituent of Tissue Extract No. 568 (''Desympatone''), Arch. Intern. Pharmacodyn. Ther. **48**: 414, 1933.
4. Wolffe, J. B., Munch, J. C., Coombs, J. N., and Digilio, V. A.: Tissue Extract No. 568. XV. Blood Pressure Response of Dogs Before and After Unilateral and Bilateral Adrenalectomies (Acute Experiments), Unpublished Mss.
5. Gley, P., and Kisthinios, N.: A propos de l'action des extraits pancreatiques sur la pression arterielle, Compt. rend. Soc. de biol., March 20, 1929.
6. Elliott, A. H., and Nuzum, F. R.: The Pharmacologic Properties of an Insulin-Free Extract of Pancreas and the Circulatory Hormone of Frey, J. Pharmacol. & Exper. Therap. **43**: 463-475, 1931.
7. Wolffe, J. B.: Angina Pectoris: Its Treatment With Insulin-Free Pancreatic Extract (''Desympatone''), Med. World, June, 1933.
8. Wolffe, J. B.: Angina Pectoris: Its Treatment With Insulin-Free Pancreatic Extract Tissue Extract No. 568 (''Desympatone''), Delaware State M. J. **7**: 123, 1935.
9. Frey, E. K., Kraut, H., and Bauer, E.: Ueber ein neues Kreislaufhormon, Ztschr. Physiol. Chem. **175**: 97, 1928.
10. Vaquez, H., Giroux, R., and Kisthinios, N.: De l'action de certain extraits pancreatique dans le traitement de l'algine de poitrine, Presse méd. **27**: 277, 1929.
11. Nuzum, F. R., and Elliott, A. H.: Pancreatic Extract in the Treatment of Angina Pectoris and Intermittent Claudication, Arch. Int. Med. **49**: 1007, 1932.
12. Chabanier, H., and Truchot, P.: Hypertension Arterielle avec syndrome d'insuffisance du coeur gauche traite et amelioree par un extraite d'origine pancreatique, Bull. Soc. franç. d'urol. **8**: 313, 1929.

13. Chabanier, H., and Truchot, P.: Pancreas Therapy in Arterial Hypertension With Cardiac Insufficiency, J. d'urol. **28**: 405, 1929.
14. Farini and Roncato: Sull azione ipotensiva dell pancreas, Lo sperimentale **64**: 46, 1910.
15. Gley, P., and Kisthinios, N.: Hypotensive Substance, Distinct From Insulin, Compt. rend. Soc. de biol. **100**: 971, 1929.
16. Kervarec and Enachesco: L'action des extraites pancreatiques prives d'insuline dans l'hypertension arterielle, Prog. med., Feb. 1, 1930, thru Ref. 19.
17. Kisthinios, N.: Action des extraites pancreatiques (insuline) sur l'hypertension arterielle, Bull. Acad. Med., Nov. 27, 1928.
18. Kisthinios, N., and Gomez, D. N.: Le traitement de l'hypertension arterielle, La Vie Med., 1930, thru Ref. 19.
19. Giroux, R., and Kisthinios, N.: Les Extraites Pancreatiques Desinsulines en Therapeutique, Paris, 1931, Publ. Masson, 126 pages.

ADRENALIN NECROSIS AFTER SYMPATHECTOMY*

A Study of Some Factors Involved

K. Leora McCloskey, M.D., Frank W. Co Tui, M.D., John Mulholland, M.D., and Arthur M. Wright, M.D., New York, N. Y.

INTRODUCTION

IN A preliminary report[1] these authors noted local tissue necrosis following injection of adrenalin hydrochloride into the hind extremities of dogs whose sympathetic nerve supply to those areas had been destroyed. This necrosis was manifest as a circumscribed area of dry gangrene, coming on within twenty-four hours after injection, and followed in about three days by slough. Subsequent healing was rapid unless the necrosis was extensive. Fig. 1 shows a typical sloughed area. Further studies have defined more clearly: (a) the dosage factor; (b) the relative susceptibility of sympathectomized areas at different levels in the extremity; (c) the effect of the environmental temperature; (d) the duration or permanence of this increased sensitiveness to adrenalin.

PROCEDURE

The method described in the previous series of experiments was repeated in this series. Briefly, it is as follows:

1. The lumbar sympathetic chain and ganglia on one side were removed. This operation destroyed the vasoconstrictor nerves to the hind extremity. The unoperated side was used as a control.

2. Control injections were made as follows:

a. Subcutaneous injection of sterile physiological saline into all four extremities.

b. Subcutaneous injection of adrenalin hydrochloride into the fore extremities.

*From the Laboratory of Experimental Surgery, Department of Surgery New York University College of Medicine.

Received for publication, March 25, 1936.

Aided by a Grant from the American Medical Association Committee on Scientific Research.

c. Subcutaneous injection of adrenalin hydrochloride into the hind extremity whose sympathetic nerve supply was intact.

d. Injection of adrenalin into corresponding areas in unoperated animals and then exposure of the animals to cold.

THE DOSAGE FACTOR

In the original experiments 1 c.c. of 1:1000 adrenalin hydrochloride was injected subcutaneously. In two cases this dose produced massive sloughs exposing the knee joint and femur respectively. One of these dogs died; the other was destroyed. Later 0.5 c.c. of 1:1000 adrenalin solution was found sufficient to produce a local necrosis of lesser extent in other animals. The question of whether there was a "threshold necrotizing dose" then arose. Injections of various dilutions of adrenalin were made and the results are shown in Table I.

When a series of injections was made at short intervals, as in Dog 28, high dilutions were used first, followed by lower dilutions. Injected areas were chosen 4 cm. or more apart to avoid overlapping.

As shown in Table I, the smallest effective dose was found to be 1 c.c. of 1:4000. Not all dogs were affected by this dose, but higher dilutions were never found to produce necrosis.

TABLE I

THE EFFECT OF VARIOUS DILUTIONS OF ADRENALIN UPON NECROSIS IN SYMPATHECTOMIZED EXTREMITIES

DOG	DILUTION OF ADRENALIN HCl (1 C.C. SUBCUTANEOUSLY)	NECROSIS	DIAMETER OF NECROTIC AREA CM.
28	1:20,000	None	
	1:10,000	None	
	1: 5,000	None	
	1: 4,000	Present	1.0
3	1: 5,000	None	
	1: 4,000	Present	1.5
14	1: 5,000	None	
	1: 4,000	Present	2.5
22	1: 4,000	Present	2.5
24	1: 4,000	None	
	1: 3,000	Present	1.0
61	1: 4,000	Present	0.8
99	1: 4,000	Present	1.0
200	1: 4,000	Present	0.6
8	1: 4,000	Present	0.8

RELATIVE SUSCEPTIBILITY OF SYMPATHECTOMIZED AREAS AT DIFFERENT LEVELS

Since a small dose of adrenalin, effective at one area of the thigh, sometimes failed to produce necrosis at a higher level of the thigh, it was decided to test the ineffective dose at a still lower level, namely, in the leg. Table II is a typical record of such an experiment and shows the greater sensitivity of the tissues of the leg than those of the thigh to adrenalin.

This observation is significant in view of other evidences that distal parts have a relatively less efficient blood exchange than more proximal parts. Some of these evidences are the development of dry gangrene in extremities, and the

TABLE II

SHOWING THE RELATIVE SENSITIVITY OF THIGH AND LEG

DOG	DILUTION OF ADRENALIN HCl (1 C.C. SUBCUTANEOUSLY)	SITE OF INJECTION	NECROSIS	DIAMETER OF NECROTIC AREA CM.
2-0	1:4,000	Thigh	None	
	1:3,000	Thigh	Present	2.0
	1:4,000	Leg	Present	1.0

more rapid cooling and freezing of distal areas exposed to cold. Whether this evidence of poorer blood supply is due to such physical factors as the more rapid cooling of areas of relatively less volume, to the smaller caliber of the blood vessels, to a poorer vasodilator mechanism, or to a combination of all or a part of these factors is not clear.

EFFECT OF VARIATIONS IN ENVIRONMENTAL TEMPERATURE

The experiments described in our preliminary report, and those listed in Table I of the present report were performed in the winter months. To our surprise, most animals injected during the summer months failed to show necrosis when similarly injected with a 1:1000 solution. It then occurred to us that the environmental temperature might be a factor. Table III represents our experiments to test this possibility. The animal house is normally maintained during cold weather at temperatures of from 68° to 78° F. over the twenty-four-hour period. Animals failing to develop adrenalin necrosis at those temperatures were handled as follows: during cold weather, animals to be injected were tied in an adjoining unheated room for one or two hours. The temperature of this room was checked by a thermometer. It was often cold enough to produce shivering and erection of hair except over the sympathectomized areas. After the two-hour period, a subcutaneous injection of adrenalin hydrochloride was made. The animals remained in the cold room one and one-half or two

TABLE III

EFFECT OF COLD ON ADRENALIN NECROSIS IN SYMPATHECTOMIZED EXTREMITIES

	EFFECT OF WARMTH			EFFECT OF COLD		
DOG	TEMPERATURE ° F.	DILUTION OF ADRENALIN HCl (1 C.C. SUBCUTANEOUSLY)	NECROSIS	TEMPERATURE ° F.	DILUTION OF ADRENALIN HCl (1 C.C. SUBCUTANEOUSLY)	NECROSIS DIAM. CM.
7	78	1:2,500	None			
	67	1:1,000	None	36	1:1,000	2.0
5	78	1:3,000	None			
	83	1:2,000	None			
	67	1:1,000	None	41	1:1,000	2.0
66	78	1:4,000	None			
	75	1:2,000	None			
	72	1:1,000	None	41	1:1,000	2.5
25	83	1:2,000	None			
	69	1:1,000	None	27	1:1,000	1.0
Normal Dog 80				27	1:1,000	None
Normal Dog 100				41	1:1,000	None

hours after injection. Under such conditions the operated animals developed areas of necrosis on the sympathectomized side. Unoperated animals showed no necrosis under the same conditions.

To the above list may be added 2 animals from the earlier series of experiments. Both had shown sloughs in areas injected with 1 c.c. of 1:4000 adrenalin subcutaneously into the thigh when the environmental temperature was 75° F. After healing of the sloughed areas both animals were reinjected. One of the

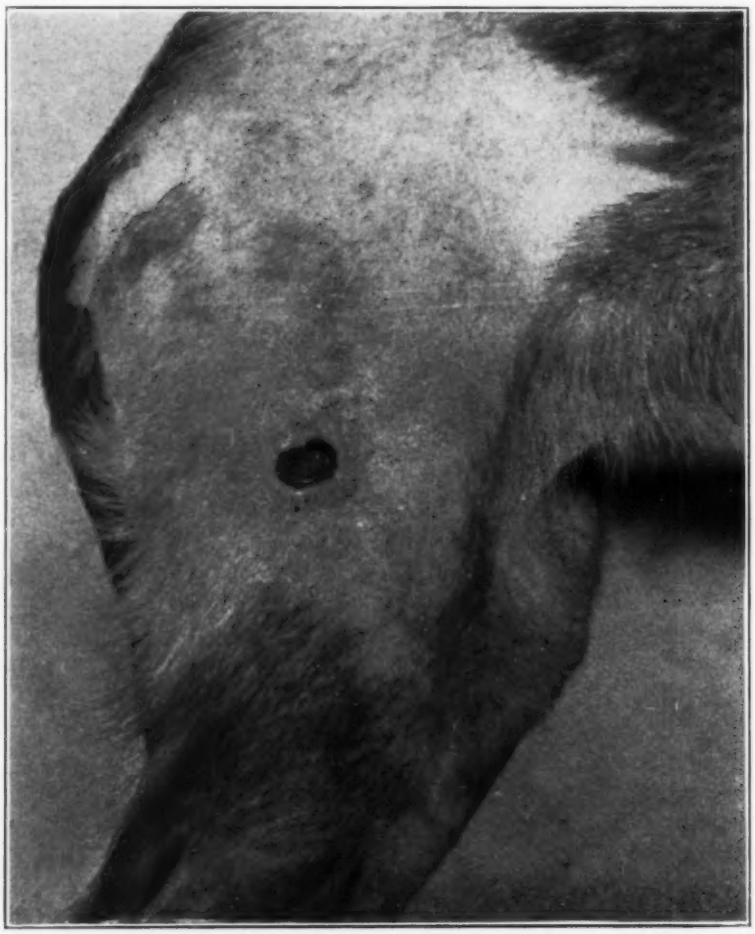

Fig. 1.—Adrenalin necrosis in sympathectomized extremity.

animals received the second injection in the thigh at a point below the site of the original injection. The other animal received the second injection in the leg in an area shown to be the more sensitive. The second dose was the same as the first (1 c.c. of 1:4000). Both of these animals were placed in small cages in front of a gas heater for two hours. Neither of the animals showed necrosis.

DURATION OF SUSCEPTIBILITY

Dog 99 was operated upon in February, 1934. In January, 1935, an adrenalin injection was followed by necrosis and slough. The photograph (Fig. 1)

of this animal was taken at that time. In January, 1936, injection of the same amount of adrenalin into the leg was again followed by necrosis. Unfortunately for more conclusive data, this is the only animal available two years after operation. In this dog, at least, the increased sensitiveness remained two years after sympathectomy.

DISCUSSION

The phenomenon of local necrosis following injection of adrenalin into sympathectomized areas is analogous to increased sensitivity of the denervated heart, iris, and intestinal muscle as cited in the previous report. This necrosis is apparently produced by a prolonged vasoconstriction in the areas injected.

The influence of the environmental temperature upon production of the necrosis suggests the mechanism. The nonsympathetic dorsal-root dilator fibers are distributed primarily to the skin and viscera.[2] The sympathetic dilators are distributed chiefly to skeletal muscle,[3, 4] and hence may be disregarded as a factor in this phenomenon. In all of our operated animals the nonsympathetic dorsal-root dilator fibers to the skin were intact. These apparently were able in some cases to overcome the constricting influence of the injected adrenalin. But in animals exposed to low temperatures the action of the nonsympathetic dilators to the skin was inhibited. This allows the abnormal vasoconstriction caused by adrenalin sufficiently to reduce the blood supply to the skin of the injected area to produce necrosis and dry gangrene. Heat, on the other hand, stimulates the nonsympathetic dorsal-root vasodilators, counteracts the excessive vasoconstricting action of adrenalin and prevents necrosis.

SUMMARY AND CONCLUSIONS

It is shown in the present report that (1) the highest dilution of adrenalin hydrochloride in 1 c.c. doses which causes necrosis in a sympathectomized area is 1:4000; (2) the distal skin area of the extremity is relatively more sensitive; (3) cold promotes the production of necrosis, whereas warmth prevents it; (4) increased sensitivity (after sympathectomy) to adrenalin lasts at least two years.

The rôle of the vasodilators in the causation and prevention of this necrosis is discussed.

REFERENCES

1. Wright, Mulholland, McCloskey, and Co Tui: Local Adrenalin Effect After Sympathectomy, J. LAB. & CLIN. MED. **20**: 947, 1935.
2. Bayliss: Physiology, ed. 4, New York, 1931, Longmans, Green & Co.
3. Cannon, W. B., and Rosenblueth, A.: Studies on Conditions of Activity in Endocrine Organs. Sympathin E and Sympathin I, Am. J. Physiol. **104**: 557, 1933.
4. Rosenblueth, A., and Cannon, W. B.: Some Circulatory Phenomena Disclosed by Ergotoxine, Am. J. Physiol. **105**: 373, 1933.

THE EFFECT OF CERTAIN TYPES OF STIMULI ON THE DEFENSE MECHANISM OF THE ANIMAL ORGANISM*

RUTH WESTLUND JUNG, M.S., PH.D., CHICAGO, ILL.

THE beneficial effects of many therapeutic procedures are not completely understood. In particular, the results of physiotherapy are only partly explained by the hyperemia and other physiologic manifestations. For this reason it is important to inquire whether these measures result in immunologic changes demonstrable by the usual laboratory methods of estimation. The immunologic effects of exercise and fatigue especially have significant implications in the field of preventive medicine.

The object of the investigation reported here was to see whether certain forms of treatment could be shown to influence those mechanisms for which there are convenient quantitative means of measurement. The types of treatment studied were as follows: heat, cold, alternate heat and cold, and exercise. In addition the action of the neutral salts of iodides and thiocyanates were examined because their position in the lyotropic series of Hofmeister suggests that their presence in the blood stream or cells of the body might affect immunologic reactions. The effects of these agents were determined upon sedimentation rates, agglutinin titer, hemolytic amboceptor titer, complement titer, normal opsonins, phagocytic power of leucocytes, and total white blood cell counts.

METHODS

1. *Sedimentation Rates.*—A review by Westergren[1] contains 324 references on the suspension stability of the red cells in man and its significance. The normal rates as determined by Fåhraeus,[2] and generally accepted, are as follows: The blood of males shows a rate of from 1 to 3 mm. and that of females from 4 to 7 mm. during the first hour. A rate of 30 mm. and above is considered a very marked reaction. Normal sedimentation rates in animals have not been definitely established. Rees Walton[3] states that the stability varies in different animals, each having its characteristic rate. Whether he meant that the variation occurred among different species or among different individuals of the same species is not clear. It is well known that certain factors, such as pregnancy and infection, influence the rate in man, and it is likely that similar changes take place in animals. The explanation of these variations is not entirely plain. If the theory is accepted that increases in rate are due to the increased globulin and fibrinogen content of the plasma, and since it is generally agreed that globulins play a part in immunologic reactions, then it is reasonable to assume that sedimentation rate changes may be important in immunity.

The following method, based on that of Fåhraeus, was used. A tuberculin syringe (1 c.c.) was filled to the 20 mark with 3.7 per cent sodium citrate solu-

*From the Department of Bacteriology, Northwestern University Medical School.
Received for publication, April 4, 1936.

tion; this represented 0.2 c.c. Then blood was drawn into the syringe up to the 1 c.c. mark. This gave four volumes of blood and one of sodium citrate. This mixture was discharged into a test tube and a pipette (1 c.c. with 0.25 mm. bore) was inserted into the tube. The mixture was then drawn up into the pipette as far as it would go without drawing in any air bubbles. Without releasing the finger, the pipette containing the mixture was inserted into a small tube (10 by 1 cm.) containing about 2 c.c. of mercury. Tubes thus prepared were set in a rack and maintained in the vertical position. The mercury served to keep the level of the mixture constant. The pipettes were incubated at room temperature and hourly readings were made. The reading at the end of one hour is the only one considered here. It was observed that in dog and rabbit blood sedimentation was practically complete in six hours. If kept twenty-four hours the blood is liable to undergo change and readings are no longer accurate.

Out of 14 determinations on 4 apparently healthy female dogs, an average of 3 mm. was obtained. Individual variations occurred as shown below.

DOG	RATES IN SUCCESSIVE DETERMINATIONS			
N5	1	5	2	7
N8	1	1	1	4
N6	6	4	7	4
3	4	3		

One dog, N2, showed rates on 4 different occasions between January and April of 10, 10, 20, 43 mm. Shortly after the last rate had been determined the dog gave birth to pups. No doubt the change in rate was due to pregnancy. Another dog, N4, a male, gave rates of 18, 11, 12 mm. This dog had an eye infection and was probably suffering from distemper, as it was found dead in its cage shortly after these rates were determined. Of 20 determinations on 4 rabbits, an average sedimentation rate of 1.2 mm. was obtained with a minimum of zero and a maximum of 4 mm.

2. *Agglutinin Titer.*—The quantitative reaction is valuable because of the comparative ease and regularity with which agglutinins in the serum can be estimated. Although the most important application of this test is in diagnosis its utilization as a measure of immunity has value. The functional rôle of agglutinins has been reviewed by Bailey,[4] who considers them closely related to opsonins and phagocytosis and believes that they play an important rôle in resistance to and recovery from infections. It is possible to stimulate the production or appearance of agglutinins by nonspecific means. As Wells[5] has stated, individuals who at one time had typhoid but whose blood no longer contains agglutinins may show a high typhoid agglutinin content when infected by some other organism, or after any sharp febrile attack. He thinks it also highly possible that many therapeutic agents may act similarly by stimulating the tissues to increased formation of antibodies.

In the agglutination test saline suspensions of twenty-four-hour agar slant cultures were used. The ordinary macroscopic test was employed. All serums from any one particular case were tested at the same time. If this was not possible a serum which had been tested before was included with the later samples.

3. *Hemolytic Amboceptor Titer.*—Hemolysins, like agglutinins, are antibodies whose presence can be easily determined. While hemolysins for sheep's red blood cells are not, as a rule, looked for when a condition of immunity is to be investigated, the reaction is probably similar to that of the bacteriolysins and no doubt the two are affected by the same types of stimuli. For this reason the effects of certain stimuli on the content of sheep cell hemolysins were investigated in a rabbit which had previously been injected with sheep's red blood cells. The presence of hemolytic amboceptor was determined in the following way: The serum was inactivated at 56° C. for thirty minutes, then mixed in varying amounts with constant amounts of guinea pig complement and a 1 per cent suspension of sheep's washed red blood cells. The tubes were incubated at 37° C. for fifteen minutes. Readings were made at the end of the incubation period and after eighteen hours in the ice box. Only the smallest amount of serum showing complete hemolysis with 0.1 c.c. of fresh guinea pig serum (1:4 dil.) is reported here.

4. *Complement Titer.*—According to Muir[6] complement is one of the chief mechanisms which contribute to acquired antimicrobic immunity. In the case of natural lytic action exhibited by fresh serum, complement is an essential factor. Further, complement contributes to intracellular destruction of organisms by rendering them susceptible to phagocytosis, the opsonic action of normal serum depending on the presence of this substance. Complement function is bound up with the protein constituents of the blood plasma, and any stimulus affecting the proteins of the blood might conceivably alter the complement content. The amount of complement in serum is easily measured and changes which might occur under various conditions should become apparent when tested by the in vitro method usually employed. The tubes were incubated thirty minutes at 37° C. and readings were made at the end of that time and after eighteen to twenty-four hours in the ice box. Only the smallest amount of serum showing complete hemolysis is reported in the tables.

5. *Opsonin Titer.*—Establishment of immunity in a great many infections is accompanied by an increase in the opsonic properties of the serum. Ledingham[7] considers the development of opsonic antibodies the most important factor in antibacterial immunity. Physical and chemical agents are known to affect opsonins. Calcium chloride, for instance, while stimulating the phagocytic function of leucocytes, inhibits its opsonic effects.[5] Although the opsonic test is criticized as being inaccurate, it should be possible to control the factors influencing the test so that reliable data can be obtained. The technic of the method which has been used in these experiments has been described elsewhere[8] and need not be repeated here except to reiterate the observation that the percentage of phagocytosis observed is dependent on the number of leucocytes used in the test and that this point must be considered when suspensions containing different numbers of leucocytes are employed. The opsonic index has been calculated in many instances.

6. *Phagocytic Property of Polymorphonuclear Leucocytes.*—In the actual defense against infections the cells of the body are probably more important than the fluids.[5] The close association of active phagocytosis with a state of immunity is of fundamental nature, and thus conditions regulating phagocytic

action constitute an all-important problem.[6] There appears to be definite evidence that functional changes in leucocytes do occur both in health and disease, and the functional activity of leucocytes cannot be regarded as a constant quantity.[7] It seems possible that agents changing the antibody content of the serum might also change the functional activity of the circulating leucocytes.

The technic for conducting the phagocytic test is essentially the same as that for determining the opsonic content, with the exception that for the phagocytic test the same serum, or at least an untreated normal serum, is used throughout with the treated cells.

7. *Leucocyte Count.*—The importance of leucocytes in phagocytosis is well known and an increase in the number of such cells in the circulating blood or in inflammatory areas implies an increased defensive activity of the body against infection. It was interesting, for this reason, to determine whether some of the stimuli used in these experiments had any effect on the number of white blood cells in the circulating blood. Counts were made in usual manner, employing a 1 per cent solution of acetic acid as diluent.

THERAPEUTIC PROCEDURES

1. *The Application of Heat and Cold* to the surface of the body are accepted physiotherapeutic procedures although little is known of their immunologic effects. Nothing has been reported on the immunologic results of these agents when applied to the exteriorized spleen. Their direct contact with this organ, probably the seat of antibody concentration, might well be expected to produce response, evidenced by increased antibody appearance in the blood stream. The spleens of two female dogs were exteriorized by the method of Barcroft and Stephens[9] and the animals allowed to stabilize for approximately two months before being used in the following experiments. Shortly after the operation Dog 1 had a purulent infection about the spleen and Dog 2 had an eye infection, diarrhea, and the spleen was large and red. Their condition was good throughout the experimental period. A series of injections of *B. paratyphosis B* was given to each dog shortly before the agglutination tests were employed in the experiments. For four months following the operation the sedimentation rates were remarkably high, then gradually decreased. The cause of the high initial rates was not determined. The rates are recorded below.

DATE	DOG 1	DATE	DOG 2
1-25	67	1-16	29
2- 6	28	1-30	20
2-10	22	2- 2	19
2-22	31	2-16	21
2-28	28	2-28	33
3-23	25	3-21	16
3-27	27	4- 3	11
3-27	18	4-10	12
4-10	11	5-18	13
5-17	7	5-23	8
5-25	8		

A. Heat: By means of a hot water bottle or an electric pad heat was applied to the exteriorized spleens. The length of the exposure was from one and one-half to four and three-fourths hours, and the range of temperature on the sur-

TABLE I

EFFECT ON BLOOD OF AGENTS APPLIED TO EXTERIORIZED SPLEEN

Dog	HEAT																				
	NO. 1										NO. 2										
Experiment	1	2		3			4			5	6	7		8	9			10			
Hours applied	1½	2½		2			4			2½	1½	2⅛		3	4¾			2½			
Av. temp. on spleen	111.0	108.3		107.8			107.5			107.4	105.3	107.5		107.0	106.6			106.9			
	COMPL.*	COMPL.	OP. INDEX	COMPL.	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX	SED. RATE	AGGL. TITER	COMPL.	OP. INDEX	COMPL.	OP. INDEX	SED. RATE	COMPL.	SED. RATE	OP. INDEX	SED. RATE	AGGL. TITER
Before	0.05	0.05	1.0	0.03	67	1.0	0.05	28	0.9	8†	160	0.05	1.0	0.10	0.8	28	0.05	19	1.2	8†	640
Just after	0.05	0.05	0.8	0.03	60	1.2	0.05	28	0.9	10	160	0.10	1.5	0.10	1.3	-	0.05	16	1.2	8	320
24 hr. after	0.05	0.05	-	0.03	60	-	0.05	26	0.7	13	160	0.10	0.8	0.10	1.3	25	0.05	18	1.0	8	640
48 hr. after	-	-	-	-	-	-	-	-	-	-	160	-	-	-	-	-	-	-	-	6	160

Dog	COLD														ALTERNATE HEAT AND COLD					
	NO. 1										NO. 2				NO. 1			NO. 2		
Experiment	11			12			13			14	15	16			17			18		
Hours applied	3			2½			2			3	2½	2			2½			1½		
	COMPL.	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX	COMPL.	OP. INDEX	AGGL. TITER	SED. RATE	OP. INDEX	COMPL.	OP. INDEX	COMPL.	SED. RATE	AGGL. TITER	COMPL.	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX
Before	0.03	25	1.2	0.05	11	0.9	-	7	640	15	1.1	0.10	1.3	0.1	13	320	0.05	22	0.7	0.05	21	0.8
Just after	0.03	24	1.1	0.05	8	1.0	-	4	640	11	1.1	0.10	1.4	0.1	-	320	0.05	28	0.7	0.05	27	0.6
24 hr. after	0.02	31	1.2	0.04	21	1.0	0.04	16	640	16	1.2	0.05	1.4	0.1	13	640	0.05	-	0.9	0.05	-	0.9
48 hr. after	-	-	-	0.03	21	0.9	-	12	-	-	-	0.04	0.9	0.1	11	640	-	-	-	-	-	-

*Smallest amount (c.c.) of serum showing complete hemolysis.
†Rates at this time were practically normal.

face of the spleen was from 105.3° F. to 111.6° F. Determinations were carried out before, just after treatment and usually twenty-four hours later and, in some cases, forty-eight hours after treatment also. Reference to Table I and Chart 2 shows that, for the most part, no changes were detectable in the immunologic functions of the blood by direct application of heat to the spleen, even though hyperemia of that organ occurred. The slight drop in agglutinin titer of Dog 2 is of doubtful moment.

B. Cold: The classic example of the immunologic effect of cold is that reported by Pasteur, Joubert and Chamberland[10] who observed an increased susceptibility to anthrax in chickens placed in cold water. Foord[11] reviews the

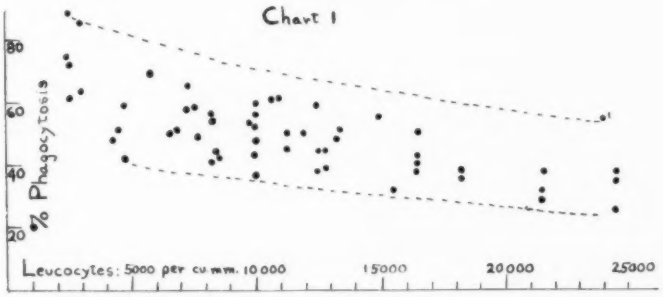

Chart 1.—Normal opsonins and phagocytes in 10 untreated dogs (No. 1, 2, 1a, 2a, 4, 5, 6, 7, 8, and 8). Cells treated with both homologous and heterologous serums. The unusually high percentage of phagocytosis represented by the point marked (*1*) was obtained in an experiment with cells from 2a and serum from 4.

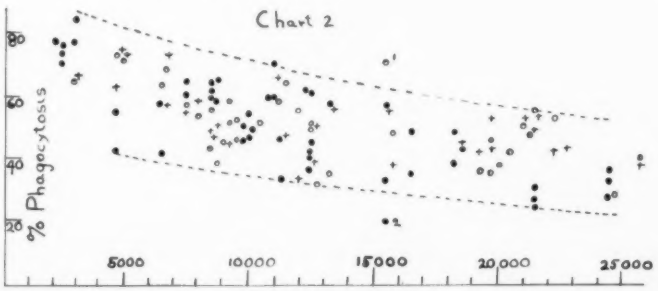

Chart 2.—Normal opsonins and phagocytes in 6 dogs (No. 1, 2, 5, 6, 7, and 8) treated with heat, cold, alternate heat and cold, NaCNS, and exercise. Dots: treated serum and untreated cells. Circles: untreated serum and treated cells. Crosses: treated serum and treated (homologous) cells. The curves are drawn in from Chart 1 only to facilitate comparison, and are not intended to convey any theoretical suggestions. Point (*1*): cells from No. 2 after exercise, serum from No. 4 (untreated). Point (*2*): cells from No. 6 (untreated), serum from No. 8 after exercise.

immunologic results of cold on the animal body and reports the conflicting results obtained by different investigators. According to Wells[12] individual cells are not greatly affected by freezing but circulatory channels are readily blocked by this cause. Exposure of the entire body of animals, he says, brings about changes in the blood similar to those of foreign protein shock.

Cold was applied to the spleen by a special ice bag, care being exercised to confine its contact to the spleen as much as possible. The time of application varied from two to three hours and blood samples were drawn just before and at different intervals after removal of the ice bag. Six experiments were per-

formed on the two dogs (Table I). The only change noted was a slight increase in the phagocytic activity of leucocytes after treatment in three of fourteen determinations. If these findings are compared with the results obtained with the total number of treated cells the variations are found to be within the normal limits (Chart 2). The slight changes in complement titer are not sufficiently marked to be considered significant.

C. Alternate Heat and Cold: Since it is the rate of stimulus which determines the intensity of a physiologic response, it was thought that by subjecting an animal to alternate heat and cold changes might occur that would not take place with heat or cold alone. Two experiments were conducted whereby heat and then cold were applied to the exteriorized spleens of Dogs 1 and 2. In the first experiment the electric pad was kept in contact with the spleen for one hour and then replaced by an ice pack for one and one-half hours. The average temperature on the surface of the spleen was 108.2° F. In the second experiment heat was applied (average temperature 104.8° F.) for three-fourths of an hour, then the ice pack for twenty-five minutes and then heat again (average temperature 106.6° F.) for twenty-five minutes.

No significant changes were noted in sedimentation rates, complement, opsonins or phagocytic property of leucocytes (Table I, Chart 2).

2. *Exercise.*—The physiologic effects of exercise on the animal organism have been reported by many workers. Fewer reports are found on the immunologic consequences although muscular fatigue and its effect on resistance to infection has been the subject of several investigations. In a review of the literature on this subject Baetjer[13] remarks on the conflicting results of various workers. Baetjer concludes that if resistance is lowered the defense mechanism of the body is altered but, since this mechanism is not well understood, thinks attempts to study this question have yielded little information.

Eighteen experiments on three rabbits and five dogs were carried out to determine whether mild exercise had any effect on the defense mechanism of the body. The dogs were put on a motor driven treadmill. No attempt was made to determine the amount of work done. Rest intervals of one to two minutes were permitted whenever the dogs appeared tired. The length of run varied with the different dogs. Another type of treadmill was devised for the rabbits. It consisted of a rotating drum of wire netting into which the rabbits were placed. The drum was attached to a motor and could be arranged to run at different speeds. It was necessary to wrap the hind feet since the animals had a tendency to drag them and produce abrasions.

Dog 2 showed an increase in the phagocytic function of its leucocytes twenty-four hours after a run lasting three hours (Chart 2). The value was normal in forty-eight hours. There was no significant change in the opsonic index (Table II). A slight decrease in opsonins was observed in Dog 8 twenty-four hours after a run lasting two hours (Table II, Chart 2). In the majority of cases no change was observed. Number 7 exhibited the most marked variation in sedimentation rate. Rabbit WS evidenced a slight increase in opsonins after six and one-half hours of exercise. The value returned to its normal level in twenty-four hours (Table III, Chart 4).

Temperature changes were evident in several instances and exhibited, for the most part a gradual rise during exercise (Table III).

Sedimentation rates in rabbits were very constant. The values were lower than those for dogs, averaging from 1 to 2 mm. Hemolytic amboceptor showed no change due to exercise.

White blood cell counts were made in one rabbit (Table IV). There appeared to be a temporary decrease followed by a slight increase. These differences may have no significance since the normal variation in the leucocyte count of rabbits is considerable, Wagoner and Custer[14] giving the figures of 4,000 to 13,000.

The agglutinin titer for *B. paratyphosis B* dropped slightly after two and one-half hours of exercise but at the end of six and one-fourth hours had risen again (Table IV).

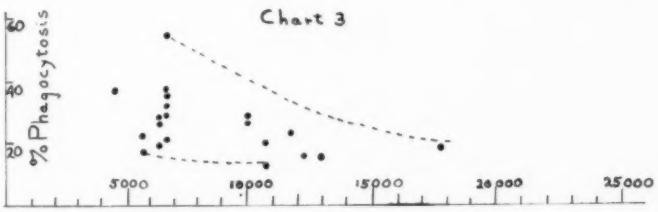

Chart 3.—Normal opsonins and phagocytes in 3 untreated rabbits (W2, W4, and WS).

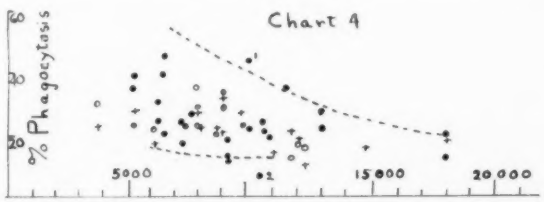

Chart 4.—Normal opsonins and phagocytes in 5 rabbits after treatment with exercise (6 experiments, rabbits W2 and WS), NaCNS (one experiment, rabbit W2), and KI and NaI (3 experiments, rabbits 2, 3, and 4). Dots: treated serum and untreated cells. Circles: untreated serum and treated cells. Crosses: treated serum and treated (homologous) cells. Point (*1*): serum from WS, after exercise tested with normal cells. Point (*2*): serum from WS after exercise tested with cells taken from WS before exercise.

In general there were no marked or constant changes in dogs or rabbits due to exercise as shown in these experiments (Tables II, III, and IV).

3. *Thiocyanates and Iodides.*—Thiocyanates and iodides are neutral salts whose position in the lyotropic series of Hofmeister suggest, as mentioned before, that their presence in the blood stream or cells of the body might influence immunologic reactions. If antibodies are globulins and if globulins are present in the cells, then the presence of thiocyanate or iodide in the blood might so change the colloidal structure of the cell or so increase the permeability of the cell wall that the globulin would enter the blood stream. The experiments reported here were designed to determine whether such results actually occur.

Gordon and Thompson[15] studied the parallel action of neutral salts on the inhibition of complement in vitro, and on the dispersion of gelatin. They noted

TABLE II

EFFECT OF EXERCISE ON BLOOD OF DOGS

Dog	NO. 1						NO. 2						NO. 5			NO. 7			NO. 8		
Experiment	1			2			3			4			5			6			7		
Hours' exercise	1			2			1/6			3			1/3			2½			2		
	COMPL.*	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX	COMPL.	SED. RATE	OP. INDEX
Before	0.02	18	1.3	0.03	27	1.5	–	33	0.9	–	11	0.9	0.10	1	1.1	0.05	15	1.0	0.05	1	1.0
Just after	0.03	16	1.3	0.03	23	1.5	0.05	28	1.2	0.10	11	0.8	0.05	2	1.2	0.05	13	1.0	0.05	1	1.0
24 hr. after	0.03	27	1.2	0.02	27	1.3	0.04	27	1.1	0.10	14	0.9	–	–	–	0.05	8	1.1	0.04	1	0.6
48 hr. after	–	27	–	0.03	27	1.0	–	–	–	0.05	18	0.9	–	–	–	0.05	6	–	0.04	–	1.8

*Smallest amount (c.c.) of serum showing complete hemolysis.

TABLE III

EFFECT OF EXERCISE ON BLOOD OF RABBITS

RABBIT	NO. W 2							NO. WS								
Experiment	8				9			10			11			12		
Hours' exercise	¼				6			1/6			1/6			¼		
	SED. RATE	AGGL. TITER	TEMP.	OP. INDEX	SED. RATE	AGGL. TITER	TEMP.	SED. RATE	HEM.* AMBO.	OP. INDEX	SED. RATE	HEM. AMBO.	OP. INDEX	SED. RATE	TEMP.	OP. INDEX
Before	4	640	104.0	1.0	1	640	102.3	2.0	0.14	1.0	0	0.18	1.0	2	102.4	1.0
Just after	–	–	–	1.1	1	640	104.4	2.0	0.16	1.2	0	0.16	0.5	2	104.0	1.2
4 hr. after	1	640	105.4	0.9	–	–	–	1.0	0.16	1.0	–	–	–	0	101.9	1.0
24 hr. after	1	640	102.8	–	1	640	103.0	1.5	0.08	–	2	0.14	–	2	102.8	–
48 hr. after	1	640	103.4	–	1	640	98.0	2.0	–	–	–	–	–	–	–	–

RABBIT	NO. WS								NO. T 4					
Experiment	13				14				15			16		17		
Hours' exercise	4				6½				7			6½		7		
	SED. RATE	TEMP.	HEM. AMBO.	OP. INDEX	SED. RATE	HEM. AMBO.	TEMP.	OP. INDEX	SED. RATE	HEM. AMBO.	TEMP.	SED. RATE	AGGL. TITER	TEMP.	SED. RATE	TEMP.	AGGL. TITER
Before	1	100.2	0.4	0.6	1	0.3	–	1.0	0	0.40	100.2	9	1280	102.6	1	102.3	1280
Just after	5	104.8	0.3	0.8	0	0.3	106.0	1.6	1	0.36	104.8	1	1280	105.0	1	104.4	2560
4 hr. after	–	–	–	–	–	–	–	0.8	–	–	–	–	–	–	–	–	–
24 hr. after	2	102.2	0.3	0.7	1	0.3	103.2	1.0	2	0.30	102.2	2	1280	103.2	1	102.0	1280
48 hr. after	5	103.0	0.4	0.9	6	0.3	102.8	–	1	0.40	103.0	1	1280	103.2	1	99.9	1280

*Smallest amount (c.c.) of serum showing complete hemolysis.

that the inhibitory power stood in the following order: CNS > I > Br = NO_3 > $SO_4/2$ ⩾ Cl, which is similar to the lyotrope series for the salt effects in the swelling of purified gelatin at low concentrations of salts. The authors consider this strong evidence in favor of the view that the inhibitory action of neutral sodium and potassium salts is due to the dispersion or peptization of the

TABLE IV

EFFECT OF EXERCISE ON BLOOD OF RABBIT (W 2)

TIME	SED. RATE	W.B.C.	TITER	TEMP.
9:40 A.M.	1	5,600	640	99.8
10:20 A.M.	1	3,700	320	103.8
11:15 A.M.	1	2,800	320	107.0
1:00 P.M.	1	7,000	320	103.2
2:00 P.M.	2	5,700	160	105.8
3:45 P.M.	0	10,000	320	102.4

Exercise was started at 9:40 A.M., stopped at 11:15, started again at 1:00 P.M., and ended at 2:00.

TABLE V

EFFECTS OF NaCNS ON THE BLOOD

20 MG. PER KILO.—SUBCUTANEOUS

	DOG 5			DOG 6			RABBIT W 2				RABBIT T 4		
	SED. RATE	COMPL.	OP. INDEX	SED. RATE	COMPL.	OP. INDEX	SED. RATE	OP. INDEX	AGGL. TITER	W.B.C.	SED. RATE	AGGL. TITER	W.B.C.
Before	5	0.05	0.7	8	0.05	1.2	1	0.7	320	–	1	1280	11,700
2 hr. after.	8	0.05	1.0	–	–	1.0	–	–	–	–	–	640	13,000
5 hr. after	–	–	–	–	–	–	–	–	–	–	1	640	10,700
24 hr. after	4	0.05	1.0	6	–	1.2	1	–	320	5700	1	640	11,900
2 days after	4	0.05	1.4	7	0.05	–	1	1.4	640	5900	1	640	11,300
3 days after	2	–	–	–	–	–	–	–	640	4400	–	–	–
4 days after	–	–	–	–	0.05	–	1	–	–	5300	1	640	13,200
5 days after	–	–	–	3	0.05	–	–	–	–	4700	–	–	–
7 days after	–	0.05	–	2	0.05	–	1	–	–	–	–	–	–
9 days after	–	–	–	–	–	–	1	–	640	6100	–	–	–
14 days after	–	–	–	–	–	–	–	–	–	–	1	320	–

protein complexes of the serum. Although gelatin is not a serum protein, the authors think it unlikely that the effect of neutral salts on osmotic pressures of serum proteins would give results very different from those of gelatin.

According to Guerlac[16] thiocyanates penetrate only very slowly into the cell like most strongly ionized salts, but the small amount which does get in is enough to bring about a definite change in the colloidal condition of the cytoplasm. The fact that sodium thiocyanate influences the aggregation of colloids in the same way as iodides[17] suggested its therapeutic use, especially in syphilis. Iodides are used principally in tertiary syphilis. They are not believed to act on the spirochetes but lead to absorption of gummatous exudations.[17] The exact reason for the beneficial effects of iodides and thiocyanates is unknown.

Tunnicliff[18] observed the effect of sodium iodide on phagocytosis. She detected an increase of approximately 10 per cent in the amount of phagocytosis in rabbit blood three hours after the intravenous injection of 0.2 c.c. of a 10 per

cent sodium iodide solution. Strubell[19] injected 3 to 5 gm. of sodium iodide into a normal male adult and showed a decrease in the opsonic index for the staphylococcus, which occurred within fifteen minutes after the injection and lasted about eight hours, returning to its normal value the next day. He does not give his method for determining the index nor any figures. Salant and Livingston[20] noted no effect on the circulation after the introduction of large amounts of sodium iodide intravenously into cats and dogs. The failure to produce any effects they attributed in part to the rapid elimination of the salt, but they also thought it probable that it was bound in some way by the lipoids of the blood, thus preventing its action on the heart and blood vessels.

The very meager data available on the immunologic effect of thiocyanates and iodides prompted a study of such effects when injected into the animal body. Experiments were performed on two dogs and three rabbits. The dogs were normal animals. Rabbits W 2 and T 4 had been immunized against a laboratory strain of *B. paratyphosus B.* Rabbit WS had been injected with sheep's red blood cells.

a. Thiocyanate: Sodium thiocyanate was injected subcutaneously or intravenously in 20 mg. per kilogram weight doses. No significant changes were observed in any case (Tables V, VI, Charts 2, 4). Rabbit W 2 exhibited a

TABLE VI

EFFECT OF NaCNS ON THE BLOOD

	20 MG. PER KILO.—INTRAVENOUS													
	DOG 5				DOG 6				RABBIT W 2			RABBIT WS		
	SED. RATE	COMPL.	OP. INDEX	W.B.C.	SED. RATE	COMPL.	OP. INDEX	W.B.C.	SED. RATE	AGGL. TITER	W.B.C.	SED. RATE	HEM. AMBO.	W.B.C.
Before	2	0.10	1.0	9,200	4	0.10	1.2	19,500	2	640	6400	1.0	0.35	12,800
1 hr. after	–	–	–	–	5	0.10	1.0	18,500	–	–	–	–	–	–
2 hr. after	2	0.10	–	10,000	–	–	–	–	–	–	–	–	–	–
3 hr. after	–	–	0.9	–	–	–	–	–	1	640	4400	–	–	–
24 hr. after	6	0.10	0.9	16,600	3	0.05	1.2	18,100	1	1280	4600	1.0	0.35	12,300
2 days after	6	0.05	–	11,000	2	0.04	–	23,000	–	–	–	2.0	0.40	11,800
3 days after	–	–	–	–	–	–	–	–	1	640	5100	2.0	0.30	8,400
4 days after	–	0.05	–	16,000	–	0.05	–	11,500	–	–	–	–	–	–
5 days after	–	–	–	–	–	–	–	–	2	320	4900	1.0	0.35	10,300
7 days after	–	–	–	–	2	–	–	–	2	320	6000	2.0	0.40	7,700
8 days after	4	0.10	–	–	2	0.10	–	22,000	–	–	–	–	–	–
10 days after	8	0.05	–	9,700	2	0.05	–	12,400	–	–	–	1.0	0.40	8,400
14 days after	2	0.10	–	11,900	1	0.10	–	12,600	1	640	4300	1.5	0.40	11,700

remarkable constancy in the number of leucocytes. Normal variations in the white blood cell counts of rabbits are wide.[14] The opsonins of Dogs C 13 and C 14, injected thirteen days previously, were compared with those of a normal dog. The three gave values quite similar, respectively 11, 8, and 10 per cent phagocytosis with human leucocytes.

b. Iodides: Three rabbits were injected with potassium made up in 10 per cent strength. Rabbit 2 received four daily injections of 0.1 gm. intraperitoneally. Rabbit 4 received 3 c.c. intravenously. Rabbit 5 was injected intravenously with 3 c.c. on the first day and with two doses of 3 c.c. each on the

second day. It died immediately after the third injection. One rabbit and one guinea pig were injected with sodium iodide. Rabbit 3 was given five daily intravenous injections of 2 per cent sodium iodide in amounts varying from 10 to 20 c.c. The rabbit died just after the fifth injection. The guinea pig received intravenously 0.1 mg. per kilogram of body weight. Intravenous injection of 0.28 gm. in 10 per cent strength produces anaphylactoid and blood changes in guinea pigs.[21]

Table VII and Chart 4 give the results of the experiments with potassium and sodium iodides. Although from the table it might be concluded that an

TABLE VII

EFFECT OF KI AND NaI ON THE COMPLEMENT TITER AND OPSONINS

	KI						NaI				
	RABBIT 2		RABBIT 4		RABBIT 5		GUINEA PIG G			RABBIT 3	
	PER CENT PHAG.	LEUCO. USED PER C.MM.	PER CENT PHAG.	LEUCO. USED PER C.MM.	PER CENT PHAG.	COMPL.	PER CENT PHAG.	LEUCO. USED PER C.MM.	COMPL.	PER CENT PHAG.	LEUCO. USED PER C.MM.
Before	21	7400	22	7000	12*	0.4	12	8800	0.15	12	8800
1 hr. after	22	7400	–	–	–	–	9	8800	0.15	9	8800
2 hr. after	–	–	23	7000	11*	0.4	–	–	–	–	–
24 hr. after	–	–	16	7000	20*	0.4	18	7600	0.15	18	8800
3 days after	39	5200	–	–	–	–	–	–	–	–	–
4 days after	35	5200	–	–	–	–	34	7600	–	34	7600
5 days after	–	–	–	–	–	–	35	7600	–	25	7600

*No count made on leucocyte suspension used in test. Control rabbit showed 19 per cent phagocytosis.

increase in opsonins occurred four and five days after treatment in Rabbit 3, if one compares these figures with those on Chart 4, in which are entered counts made on five rabbits after treatment, the figures fall within the limits of normal variation. No changes are effected by potassium iodide or sodium iodide in the two cases tested (Table VII).

SUMMARY

1. Sedimentation rates of blood were not measurably altered by exercise nor by sodium thiocyanate injections in dogs or rabbits.

2. Agglutinin content of blood serum was not significantly affected in dogs by local application of heat or cold to the surface of the spleen, nor in rabbits by exercise or sodium thiocyanate injections.

3. Complement content was not changed in dogs by local application of heat, cold, alternate heat and cold to the spleen, in dogs or rabbits by exercise and sodium thiocyanate injections or in one guinea pig by sodium iodide.

4. Hemolytic amboceptor content was not altered in rabbits by exercise or by sodium thiocyanate injections.

5. The white blood cell count was not consistently changed in dogs nor rabbits after sodium thiocyanate injections. In one rabbit tested during and after exercise there was an initial decrease in the number of leucocytes followed by a return to normal within a few hours.

6. There were no significant changes in the opsonin content of the serum of dogs or rabbits by exercise, in dogs by local application of heat, cold, alternate

heat and cold to the surface of the spleen or in dogs or rabbits injected with sodium thiocyanate. There was no change in one guinea pig injected with sodium iodide nor in rabbits injected with potassium or sodium iodides.

7. No marked alterations occurred in the phagocytic function of the leucocytes of dogs by the application of heat, cold or alternate heat and cold to the surface of the spleen. Neither mild exercise nor sodium thiocyanate produced any marked changes in the phagocytic property of leucocytes of dogs or rabbits.

CONCLUSIONS

Changes in the defense mechanism were found to be practically within the limits of normal variation as established by the methods employed. While few animals were used, in some instances the lack of measurable response did not warrant more extensive investigation.

REFERENCES

1. Westergren, A.: Die Senkungsreaktion, Ergebn. d. inn. Med. u. Kinderh. **26**: 577, 1924.
2. Fåhraeus, R. : Suspension Stability of Blood, Physiol. Rev. **9**: 241, 1929.
3. Rees Walton, A. C.: The Erythrocyte Sedimentation Test, Quart. J. Med. **2**: 79, 1933.
4. Bailey, G. H.: The Functional Rôle of Agglutinins. The Newer Knowledge of Bacteriology and Immunology, Chicago, 1928, Jordan and Falk.
5. Wells, H. G.: Chemical Aspects of Immunity, New York, 1929.
6. Muir, R.: Bactericidal Action, System of Bacteriology (a serial) **6**: 267, 1931.
7. Ledingham, J. C. G.: Natural Immunity, System of Bacteriology (a serial) **6**: 31, 1931.
8. Jung, R. W.: Correlation Between the Number of Leucocytes and Percentage Phagocytosis, Proc. Soc. Exper. Biol. & Med. **29**: 981, 1932.
9. Barcroft, J., and Stephens, J. G.: Observations Upon the Size of the Spleen, J. Physiol. **64**: 1, 1927-28.
10. Pasteur, L., Joubert, and Chamberland: La Theorie des Germes et ses Application à la Medicine et la Chirurgie, Bull. Acad. de méd. **7**: 432, 1878.
11. Foord, A. J.: Effect of Cold on Antibody Production, J. Infect. Dis. **23**: 159, 1918.
12. Wells, H. G.: Chemical Pathology, Philadelphia, 1925.
13. Baetjer, A. M.: The Effect of Muscular Fatigue Upon Resistance, Physiol. Rev. **12**: 453, 1932.
14. Wagoner, G., and Custer, R. P.: Handbook of Experimental Pathology, Baltimore, 1932.
15. Gordon, J., and Thompson, F. C.: The Parallel Action of Neutral Salts on the Inhibition of Complement and on the Dispersion of Gelatin, Brit. J. Exper. Path. **14**: 33, 1933.
16. Guerlac, H. E.: Combined Action of Ethyl Urethane and Sodium Thiocyanate on the Living Cell, Proc. Soc. Exper. Biol. & Med. **30**: 265, 1932.
17. Sollmann, T.: A Manual of Pharmacology, Philadelphia, 1922.
18. Tunnicliff, R.: Promotion of Phagocytosis by Calcium Gluconate, Sodium Iodide, Dextrose and Other Substances, J. Infect. Dis. **48**: 161, 1931.
19. Strubell: Pharmako-dynamische Probleme, Berlin. klin. Wchnschr. **2**: 2242, 1911.
20. Salant, W., and Livingston, A. E.: Effect of Iodine and Sodium Iodide on Circulation, Am. J. Physiol. **41**: 234, 1916.
21. Sollmann, T., and Hanzlik, P. J.: An Introduction to Experimental Pharmacology, Philadelphia, 1928.

THE EFFECT OF GASTRIC MOTILITY ON THE URINARY UREA CONCENTRATION TEST AND THE GLUCOSE TOLERANCE TEST*

FRANK L. APPERLY, M.A., M.D., D.Sc., AND M. KATHARINE CARY, A.B.,
RICHMOND, VA.

WHEN carrying out the urea concentration test for kidney function or the glucose tolerance test in the usual manner by giving urea or glucose by mouth we make certain assumptions, e.g., that these substances are being fed into the intestine from the stomach, are being absorbed by the gut, and passed through the liver to the blood stream at a certain "normal" rate.

It might be expected that variations from these "normal" rates would lead to alterations in the results obtained, and so to erroneous conclusions regarding kidney function or glucose metabolism. Magers,[1] finding little or no differences between the sugar tolerance curves in a number of patients when the glucose was given intraduodenally and by mouth, concluded "that the emptying rate of the stomach ordinarily has little or no effect on the glucose tolerance test." She, however, did not investigate the rates of stomach emptying. We, therefore, decided to reinvestigate the question, using both the glucose and urea tests, in a number of cases in which the rate of stomach emptying was known to vary considerably.

METHODS

Twenty-seven subjects were examined, none of whom had glycosuria or signs of kidney disease. These were divided into three classes:

1. Ten healthy people whose stomachs had been shown, by a fractional test meal within the previous six months, to empty in one hour or less.

2. Eleven patients who were found by x-ray and barium meal to have a small residue in the stomach at six hours. In all of these cases the stomach was atonic, in some instances accompanied by organic stenosis.

3. Six patients in whom there was gross retention of barium meal at six hours, following organic disease of the pylorus. It is admitted that very occasionally one finds stomachs that rapidly empty the gruel of a test meal but show a delayed emptying with the heavier barium, but as a rule, as is well known, gastric motility with the gruel meal and with the barium meal varies in the same direction.

Fifty grams of glucose and 15 gm. of urea dissolved in 100 c.c. of water were administered to each subject, who had previously been instructed to

*From the Department of Pathology, Medical College of Virginia.
Received for publication, April 14, 1936.

take neither food nor fluid for fifteen hours preceding the test. The bladder was emptied immediately before and at hourly intervals after drinking the test solution. In each specimen the percentage of urea was estimated by Van Slyke's urease method. The blood glucose was determined in capillary blood (finger prick) by MacLean's method[2] before and at half-hourly intervals after taking the test solution.

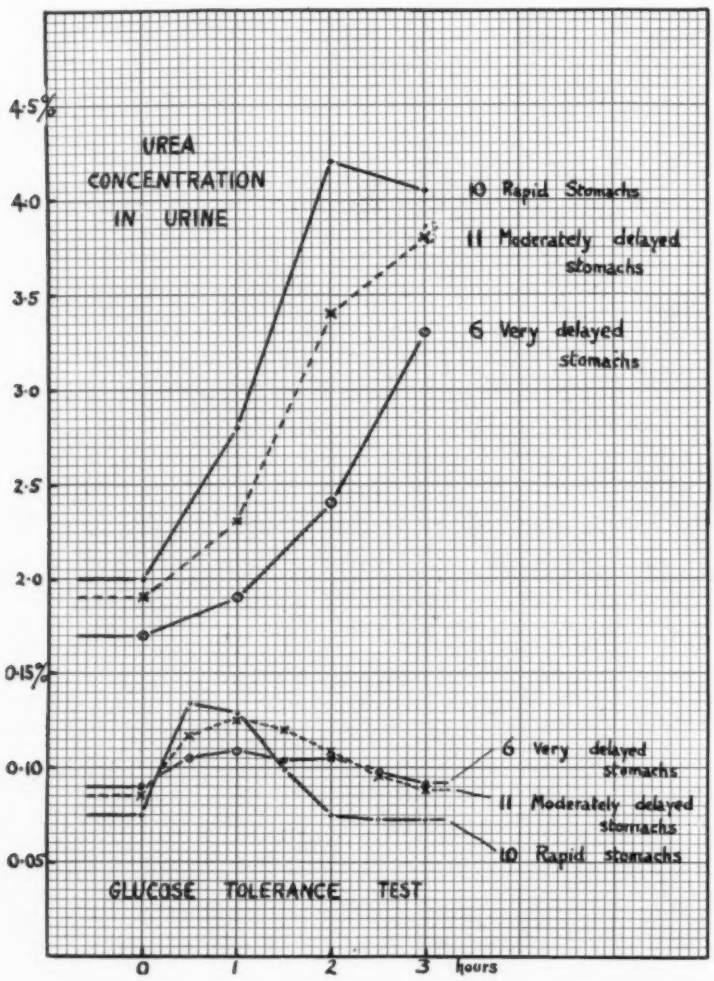

Fig. 1.

RESULTS

Only the averages of each of the groups are plotted in Fig. 1. Though the variations from the mean were much less than we had expected, there was nevertheless a fair amount of overlapping of the groups when all results were plotted.

In the case of the rapidly emptying stomachs absorption from the intestine has apparently also been rapid, resulting in a sharp rise in both the

urea and glucose concentrations in urine and blood respectively, and an early attainment of the peak in both curves. On the other hand those patients with very low gastric motility give curves with a delayed rise, the blood glucose curve being low and flat, with a marked delay in the return to the fasting level. A glance at the chart suggests that a low delayed curve would also have been found in the urea curve from the slowly emptying stomachs if the test had been carried on for a longer period.

DISCUSSION

The amount of glucose absorbed from the intestine, and therefore the peak of the blood-glucose curve, rises with the dose of glucose administered up to a maximum of 30 to 50 gm. (Hansen[3]). Larger doses have no effect on the height of the curve since, as shown by Cori,[4] absorption from the intestine cannot surpass this maximum rate. The only effect of larger doses is to prolong the time of absorption. It would appear, however, that slowly emptying stomachs feed glucose into the intestine in amounts less than the maximum absorption rate. The peaks of the curves, therefore, are lower than those found with rapidly emptying stomachs.

It has further been shown that the mechanism for the utilization of glucose "is accelerated as the blood sugar rises higher until, at the peak of the curve, utilization rate overtakes, and then surpasses, the absorption rate, in spite of the fact that the absorption may still remain at its maximum." (Peters and Van Slyke.[5]) We would therefore expect a more rapid and greater fall to subnormal in the high-peak sugar curve associated with the rapidly emptying stomach.

At present we can give no explanation of the low fasting blood glucose in those people with the unusually rapidly emptying stomachs. We would suggest, however, that the low fasting blood sugar, by increasing gastric tonus and activity (Buletao and Carlson[6]) brings about the increased motility.

Turning now to the question of urea concentration in urine, we find that, according to Gréhant[7] and Harrison,[8] the ratio $\frac{\text{urine urea concentration}}{\text{blood urea concentration}}$ or $\left(\frac{U}{B}\right)$ has a practically constant value of about fifty in normal individuals, provided the subjects have been deprived of water for twelve hours or more in order to assure a urine volume of less than 150 c.c. per hour. As our subjects drank no fluid for fifteen hours, and in no case did the volume of urine reach 100 c.c., we feel justified in using this ratio as a basis for argument. As none of our subjects had a urinary urea concentration of less than 2 per cent at two or three hours, we can also assume, according to MacLean,[2] that none had kidney disease. We can therefore deduce, from the above considerations, that the lower urinary urea curves simply mirror correspondingly lower blood urea concentrations, and that the latter in their turn are due to smaller amounts of urea being fed into the intestine from slowly emptying stomachs.

One further fact should be noticed. On the whole the patients with slowly emptying stomachs were older than those with rapid emptying, the average ages being forty-two and twenty-eight years, respectively. This factor might

account for part of the differences noted in the urea concentration test. In the glucose tolerance test, however, Hale-White and Payne[9] have shown that the height of the curve, in general, increases with age. Allowance for the age factor in our delayed motility groups might be expected, therefore, to lower still further the curves found in this group, and thus accentuate the differences observed.

CONCLUSIONS

Variations in the urinary urea concentration and glucose tolerance tests from the "normal" or healthy average may be brought about by alterations in gastric motility. The operation of the latter factor should therefore be kept in mind when such variations occur in patients with gastric symptoms.

REFERENCES

1. Magers, E. J.: Comparison of Blood Sugar Curves When Glucose Is Given Orally and Intraduodenally, J. LAB. & CLIN. MED. **19**: 608, 1934.
2. MacLean, H., and deWesselow, O. L. V.: Estimation of Sugar Tolerance, Quart. J. Med. **14**: 103, 1921.
3. Hansen, K. M.: Investigations on Blood Sugar in Man: Conditions of Oscillations, Rise and Distribution, Acta méd. Scandinav. Supp. **4**: 1, 1923.
4. Cori, C. F.: Fate of Sugar in Animal Body: Rate of Absorption of Hexoses and Pentoses From Intestinal Tract, J. Biol. Chem. **66**: 691, 1926.
5. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry: Interpretations **1**: Baltimore, 1932, Williams & Wilkins Co., p. 118.
6. Buletao, E., and Carlson, A. J.: Contributions to the Physiology of the Stomach: Influence of Experimental Changes in Blood Sugar Level on Gastric Hunger Contractions, Am. J. Physiol. **69**: 107, 1924.
7. Gréhant, N.: Mesure de l'activité physiologique des reins par le dosage de l'urée dans le sang et dans l'urine, J. de Physiol. et de path. gén. **6**: 1, 1904.
8. Harrison, G. A.: Urea Tests of Renal Function, Brit. J. Exper. Path. **3**: 28, 1922.
9. Hale-White R., and Payne, W. W.: Dextrose Tolerance Curve in Health, Quart. J. Med. **19**: 393, 1926.

THE FORMATION OF INDOL IN URINE*

STUART L. VAUGHAN, M.D., PH.D., BUFFALO, N. Y.

IN 1932 the author[1] reported the results of an attempt to detect free indol in urine. Using a test that was sufficiently sensitive to detect the substance in concentrations lower than one part in 800,000, none was demonstrated in any sample from 147 subjects, including normal individuals, patients in which there was disturbance of liver function, those in which there was marked intestinal putrefaction and those suffering from a variety of diseases.

Forbes and Neale[2] have reviewed the literature on indoluria recently and have reported experiments of their own. Their tests on the urine of fifty normal subjects were also negative except in one instance, but positive results were obtained in samples from patients suffering from a large variety of apparently unrelated clinical conditions.

The chance observation described below incited a reinvestigation of the subject. While the results substantiated my previous findings, as far as freshly voided urine is concerned, the presence of a substance that is readily converted into indol was demonstrated in the urine of both normal and diseased subjects.

A patient, during the course of a gastrointestinal x-ray study, suddenly experienced excruciating pain in the region of the bladder. Shortly afterward he passed some urine that was very cloudy and foul smelling. It was thought that a communication between the intestine and the bladder had become formed. As one of the steps taken to establish this diagnosis the urine was examined for indol on the assumption that the presence of this substance would indicate admixture with feces. A strongly positive reaction was obtained. However, the test was not made until twenty-four hours after the sample was collected and since large numbers of bacteria *(Bacterium coli)* were present the exact significance seemed questionable.

Samples of such age or degree of contamination had not been examined, and it was considered possible that the bacterial contamination had influenced the finding. Therefore, the following experiments were carried out:

I. A series of samples of urine was collected from normal subjects and hospital patients at random. Each sample was divided into two parts immediately. One part was filtered through a Seitz filter to insure sterility. After testing for indol† the parts were subdivided further and one portion of each

*From the Buffalo General Hospital and the University of Buffalo Medical School.
Received for publication, April 22, 1936.

†The indol test used in these experiments is described as follows: To a small agglutination tube is added 3 c.c. of the test substance and 1.5 c.c. of petroleum ether. The tube is inverted 50 times to insure as nearly as practicable uniform extraction of the indol. One cubic centimeter of the clear ether layer is then transferred to a second tube and 0.5 c.c. of Ehrlich's reagent added. The content of the tube is then agitated by tapping with the finger until the maximum color is produced in the lower layer.

of the original parts was inoculated with a stock strain of *Bacterium coli*. All four portions were incubated at room temperature and tests for indol were made after twenty-four hours and five days.

The following results were obtained: All tests for indol on the fresh samples were negative. The filtered, uninoculated samples remained negative throughout the duration of the experiment. Of the unfiltered, uninoculated portions, 75 per cent were positive after twenty-four hours and 83 per cent were positive after five days. All of the samples showed a growth of *Bacterium coli*. Of the inoculated samples both filtered and unfiltered, 83 per cent were positive for indol in twenty-four hours.

The intensity of the color reaction varied greatly from sample to sample. The desirability of a quantitative test was apparent. Because indol itself is utilized to some extent by bacteria as shown by Logie,[3] and further substantiated by the author, direct quantitation of this substance was impossible but comparative values could be calculated roughly by the procedure cited below.

II. Serial dilutions of pure tryptophan in physiologic salt solution ranging in concentration from 1 in 1,000 to 1 in 4,000,000 and a series of samples of fresh urine from twelve hospital patients selected at random were treated uniformly with *Bacterium coli* by a technic to be described elsewhere. The intensity of color obtained in the test for indol on the urine samples was compared with that obtained in the graded series of tryptophan solutions.

The results were as follows: In four the color was greater than that given by the 1 in 1,000 solution of tryptophan (0.1 per cent). In six the color could be compared with that of tryptophan dilutions varying from 1 in 10,000 (0.01 per cent) to 1 in 400,000 (0.0025 per cent). In two the test was negative.

III. The third experiment was conducted for the purpose of comparing the intensity of the indol reactions with the amounts of indican present. This seemed indicated because of the intimate relationship between the absorption of the products of intestinal putrefaction including indol and the excretion of indican. The test for indican was the usual one employing Obermayer's reagent and chloroform.

No correlation between the two sets of results could be demonstrated. Some samples gave a strong test for indican and a negative test for indol and vice versa.

SUMMARY AND DISCUSSION

The results of the present experiments substantiate the previous finding that free indol as such is rarely, if ever, found in urine by the methods employed, provided the tests are made on freshly voided samples. Since the method used involved minimal manipulation of samples and since it would determine very low concentrations of indol, this conclusion appears to be justified in spite of the experiments of Forbes and Neale referred to above, who, after steam distillation of urine from acid solution, obtained indol in various pathologic conditions. The author feels that their results which seem to be of pathologic significance, denote the presence, not of free indol, but rather of a relatively unstable precursor of that substance.

However, urine of the majority of subjects examined, both normal and diseased, contained a substance that was converted into indol or some compound with properties closely resembling those of indol readily by the action of *Bacterium coli*. What is the probable nature of this substance?

Since it occurred with equal frequency in urine from normal and pathologic subjects, it is almost certainly different from that described by Forbes and Neale, for the latter was present only in material from diseased individuals.

As no relationship was found between the amount of this compound and of indican contained in various specimens of urine, it is probable that the former is not absorbed from putrefying material in the intestinal tract but has some other source. Many experiments upon the metabolism of *Bacterium coli* show that this organism does not form indol from proteins or peptides, but does form it from tryptophan.[4] Indeed, as far as is known free tryptophan is the only compound which can serve as a source for indol in the metabolism of this organism. Since it is known that amino acids occur normally in urine, it seems most probable, therefore, that tryptophan was the compound demonstrated in the experiments upon urine described above.

The proof of this identity cannot be considered complete, for some compound more or less closely related to tryptophan may be formed from that amino acid in metabolism and be excreted in urine.

However, as far as the author could determine no compound has yet been described which will give positive results with the test used in these studies.

CONCLUSIONS

1. The experiments reported substantiate the previous finding that free indol is rarely, if ever, present in urine of either normal or diseased subjects if the tests are made on freshly voided samples.

2. Urine of the majority of subjects contains a substance that is converted into indol readily by the action of *Bacterium coli*.

3. The substance present is probably free tryptophan.

4. The concentration of the indol formed is frequently greater than that formed from a 0.1 per cent solution of tryptophan.

REFERENCES

1. Vaughan, S. L.: The Examination of Urine for Indol, Proc. Soc. Exper. Biol. & Med. **29**: 623, 1932.
2. Forbes, J. C., and Neale, R. C.: Studies on Indoluria, J. Lab. & Clin. Med. **20**: 1017, 1935.
3. Logie, W. J.: On the Synthesis of Tryptophane by Certain Bacteria and on the Nature of Indole Formation, J. Path. & Bact. **23**: 224, 1920.
4. Topley and Wilson: The Principles of Bacteriology and Immunology, Baltimore, 1929, Wm. Wood and Co. **1**: p. 55.

LABORATORY METHODS

AN AUTOMATIC RECORDING APPARATUS FOR MEASUREMENT OF COLLOID OSMOTIC PRESSURE*

MARGARET SUMWALT, PH.D., AND EUGENE M. LANDIS, M.D., PH.D.,
PHILADELPHIA, PA.

UNTIL recently, if the colloid osmotic pressure of a solution was to be measured without change in its volume, it was necessary to make frequent observations, and appropriate adjustments of a counter-pressure. In 1934, however, Urban described an apparatus which substituted automatic control for the readings and adjustments of the observer, and which at the same time provided a continuous record of the counter-pressure. Independently of Urban, we devised an automatic and recording apparatus for the same purpose. It offers advantages in addition to those claimed by Urban; and it has been tested and found suitable for clinical measurements.

APPARATUS†

An osmometer similar in principle to that of Krogh and Nakazawa (1927) was used, with certain modifications which made it more convenient not only for our method but for general use. The osmometer chamber was a thick-walled bottomless flask of glass (*C*, Fig. 1,) with a capacity of 2 c.c. The osmometer tube (*T*), 20 cm. long and 1 mm. in inside diameter, was ground into the top of the chamber, the joint being sealed with stopcock grease and reinforced with heavy rubber bands (*RB*). The flaring base of the chamber was ground flat on the bottom, providing a plane surface to which a wet cellophane membrane (*H*) adhered perfectly, with or without vaseline. Two thicknesses of filter paper (*J*), drenched with physiologic salt solution, furnished the external solution, as in the osmometer of Krogh and Nakazawa. A perforated bronze disc (*D*) supported the membrane and filter paper. Chamber, membrane, filter paper, and disc were held together by three thumb screws (*TS*) in a pair of brass collars (*BC*), which were cushioned with rubber rings (*RR*). When the assembly was hung in a bath of 0.6 per cent NaCl solution, a bubble of air trapped in the cone of the lower collar separated the solution in the filter paper from the solution in the bath.

*From the Robinette Foundation, Hospital of the University of Pennsylvania.
Received for publication, January 20, 1936.

†Those items of equipment which we used from commercial sources are listed below with their trade names, manufacturers, and specifications. We cannot say that each is the best obtainable for the purpose since no exhaustive comparison of various products was made; it can be said, however, that each was adequate. "Cellophane," Du Pont Cellophane Co.; grades 450 or 600. Condenser: "Telephone condenser," Western Electric; 21 E, 2 M.F. Lamp: "Headlight or spotlight," Westinghouse; Mazda 1183, 50 c.p. 6-8 v., s.c. base, S 11 bulb. Motor for hammer: "Synchronous motor," Warren Telechron Co.; type B3, model 94 M 164, 60 r. p. m., 110 v., 4 w. "Photronic cell," Weston Electrical Inst. Corp.; model 594. Relay, miniature. Weston Electrical Inst. Corp.; model 534, lower limit of range 0.075 ma. Relay, power, Struthers, Dunn Inc.; C X B 51 I, coil 0.6 ma., contacts 6 v. Transformer for lamp: "Midget toy transformer," Jefferson Electric Co.; 75 w., A. C.

A photoelectric unit, the arrangement of which is shown in Fig. 2, served to detect incipient entrance of water into the osmometer. The photronic cell (*P*) was shielded from all illumination except light rays that traversed the osmometer tube (*T*). Light was furnished by a small headlight lamp (*F*) and focused by a 16 mm. microscope objective (*O*) to an image in the lumen of the tube.

Fig. 3 shows schematically the remainder of the apparatus. To control the photoelectric unit, the serum (*s*) in the osmometer tube was covered with a layer of white mineral oil (*mo*) and a layer of dilute India ink (*i*); and the ink-oil interface was placed at the focus of the light in the tube. Those light

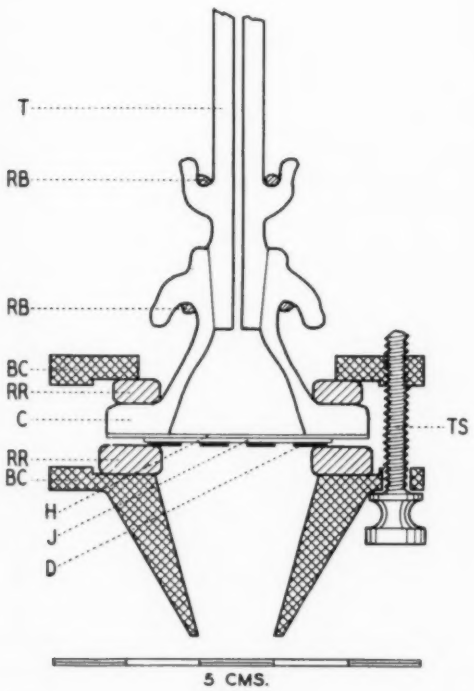

Fig. 1.

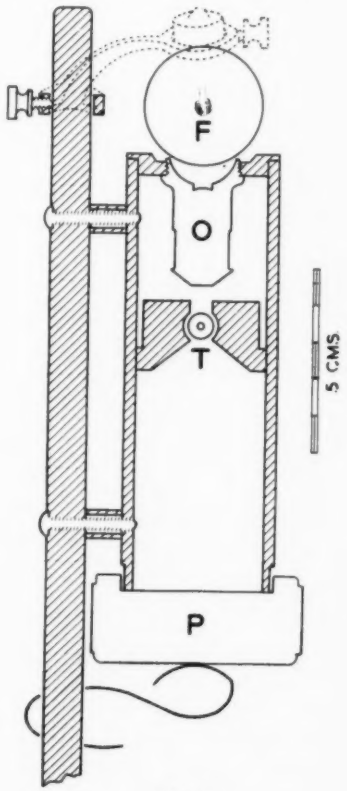

Fig. 2.

Fig. 1.—Section of the osmometer described in the text. The symbols, from the top downward, are: *T*, osmometer tube; *RB*, rubber band; *BC*, brass collar; *RR*, rubber ring; *C*, osmometer chamber; *TS*, thumb screw; *H*, membrane; *J*, filter paper; *D*, perforated metal disc.

Fig. 2.—Photoelectric unit surrounding the osmometer tube, seen in horizontal section. *F*, lamp; *O*, microscope objective; *T*, osmometer tube; *P*, photronic cell.

rays which encountered transparent mineral oil traversed the tube and reached the photronic cell; the rest were absorbed by the ink.

When increased illumination of the photronic cell signaled an incipient entrance of water into the serum in the osmometer, air pressure above the serum was increased by the electrolysis of a 25 per cent solution of NaOH between nickel electrodes in the chamber (*E*), which communicated with the top of the osmometer tube. To carry off any excess pressure generated in

this way, and to insure continuous activity of the automatic principle, gas escaped steadily from the chamber through a small leak (L). The leak was provided by tightly compressing a wisp of cotton wool in rubber tubing with a screw clip. A tube of $CaCl_2$ between the leak and the rest of the system prevented condensed water vapor from clogging the minute air passages in the cotton.

The photoelectric current was too small to produce rapid electrolysis, even when all the light focused into the osmometer tube encountered mineral oil. It was therefore amplified by two relays (R_1 and R_2). R_1 was very sensitive and controlled the current from three dry cells in circuit with the coil of R_2; while R_2, a power relay, controlled the current for electrolysis derived from a six volt storage battery. Both relays were regulated to make contact promptly when the full light beam fell upon the photronic cell; and to open when all of the light was intercepted by ink. Sticking at the relay contacts was reduced by placing across each pair a condenser in series with

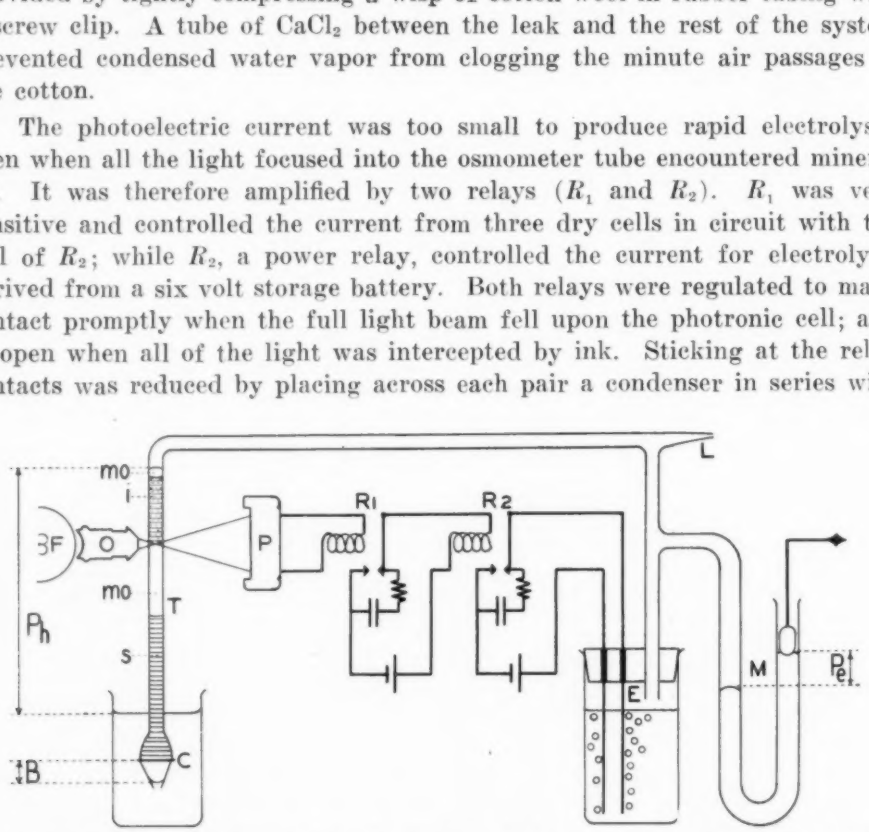

Fig. 3.—Diagram of apparatus which automatically balanced and recorded colloid osmotic pressure. From left to right: F, lamp; O, microscope objective; mo, mineral oil; i, India ink; s, serum; T, osmometer tube; C, osmometer chamber; P, photronic cell; R_1 and R_2, relays; E, electrolysis chamber; L, leak; M, mercury manometer. Three of the quantities which enter into the calculation of osmotic pressure are also indicated: P_h, hydrostatic pressure; B, the bubble correction; and P_e, the air pressure maintained by electrolysis.

a suitable resistance. To further reduce lag in their responses a small hammer operated by a telechron motor jarred the base of their panel every half second. When the sensitivity of the relays had been properly adjusted, the cycle of serum rise, photoelectric response, electrolytic increase in gas pressure, serum fall, decrease in pressure, and serum rise was completed many times a minute.

A mercury manometer of large bore (M, Fig. 3), with an ink pen supported on a glass float, recorded continually the gas pressure maintained by electrolysis. The slowly moving kymograph was provided with a time marker.

PROCEDURE

When a determination of colloid osmotic pressure was to be made, the osmometer chamber was assembled with a membrane impermeable for protein (cellophane, grade 450 or 600) and filled with serum. Insertion of the osmom-

eter tube forced serum part way up the lumen, and tilting the assembly induced it to flow out to the end. While the osmometer was slowly righted again, mineral oil followed by India ink (diluted with 15 to 20 parts of water) were fed in above the serum. If air bubbles had been avoided, and the lumen of the tube was perfectly clean and already wet, the columns of oil and ink followed the serum smoothly without fragmenting. Enough oil was introduced to keep the serum beyond the section of osmometer tube which the lamp heated. A cap of oil 2 or 3 mm. deep above the ink prevented evaporation and sticking.

The osmometer tube was then inserted in a holder, between the lamp and photronic cell, and the top of the osmometer tube was connected with the pressure system. It is theoretically desirable to adjust the apparatus so that the initial pressure equals the colloid osmotic pressure of the serum. In practice this was done approximately by clamping the osmometer tube in its support at such a height that the ink-oil interface was slightly above the beam of light before electrolysis began. At intervals during the ten minutes after electrolysis had forced the interface into the light beam, the position of the tube was changed until the recorded pressure curve appeared to be level or falling slightly. This done, the experiment ordinarily required no further attention until equilibrium was established.

To insure ample time for attaining equilibrium, the experiments were allowed to run for about twenty-two hours. The height of the column of liquid in the osmometer tube above the surface of the bath was then measured, and the kymograph record analyzed. The colloid osmotic pressure of the serum was calculated by the following equation:

$$\text{C. O. P.} = P_e + P_h - B - C$$

In which P_e is air pressure maintained by electrolysis at equilibrium, P_h is hydrostatic pressure of fluid in the osmometer tube, B is hydrostatic pressure of water in bath corresponding to the bubble trapped below the membrane, C is capillarity in the osmometer tube; the first three quantities are represented roughly in Fig. 3.

P_e was obtained from the kymograph record by measuring at regular intervals the height of the pressure curve above its zero line. Fig. 4 reproduces sections of a typical tracing, natural size; and Fig. 5 is a graph of the same experiment. A fluctuation 1 mm. high in the record corresponds to a change of 2.7 cm. water in the air pressure above the serum. The small transient changes in recorded pressure were produced by alternating bursts of electrolysis and quiescence, and the mean of these fluctuations was read. The extreme error in the measurements was equivalent to about $\pm$ 1 cm. water, when the relays were properly responsive. The large gradual drift of the recorded curve represents the approach to an equilibrium state in the osmometer. The recorded curves usually passed through a maximum, despite their apparent flatness in the first ten minutes, and then subsided. All eventually reached a level where their variations corresponded to less than 2 cm. water. Equilibrium was therefore considered established in the osmometer when the recorded air pressure had varied less than 2 cm. water in four hours. P_e was the average height in millimeters of the portion of the curve after equilibrium was established, multiplied by 2.72 to express it in centimeters of water.

P_h was the distance in centimeters from the top of the liquid in the osmometer tube to the surface of the bath measured at equilibrium. Strictly speaking, this height should be corrected for the diverse densities of the serum, oil, and ink composing the column; but the correction was negligibly small. B, the depth of the bubble trapped within the collar of the osmometer, below the outside solution, was uniformly 2.1 cm., so that the same figure was used in all observations.

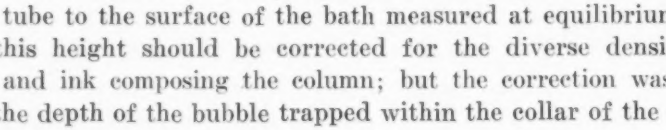

Fig. 4.—Clippings, reproduced natural size, from the kymograph record of a double experiment. The two upper lines were drawn by the floats of the manometers, from left to right. Their zero positions appear at the beginning and end of the experiment. The signals recorded on the reference line at the bottom were six minutes apart.

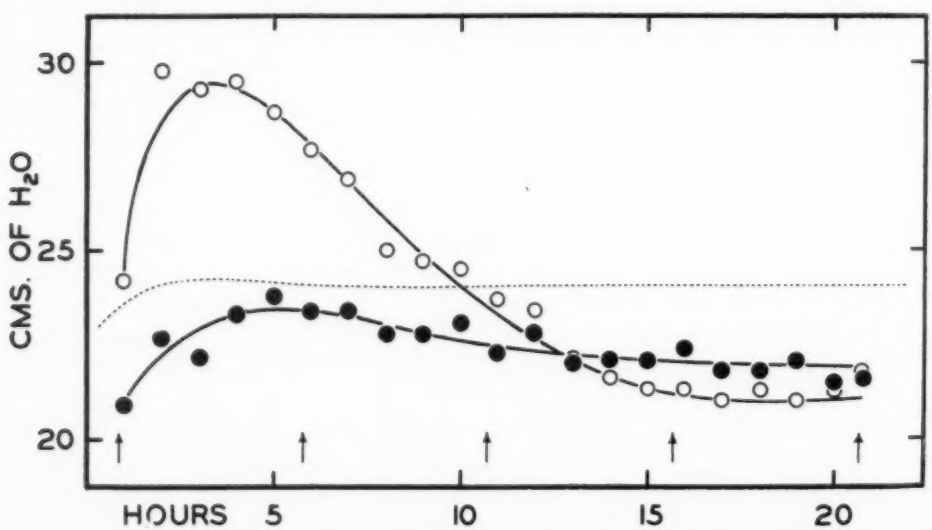

Fig. 5.—Graphic résumé of the experiment sampled in Fig. 4. Measurements of the record were corrected for hydrostatic pressure, bubble depth, and capillarity; then plotted. The solid lines are the smoothed curves for these points; hence their height at equilibrium is the colloid osmotic pressure by the automatic method. The dotted line indicates the trend of a duplicate experiment by the Wells method. The samples of records shown in Fig. 4 began at the times indicated by the arrows on Fig. 5.

To determine the length of the column of liquid which capillarity supports is impracticable by the simple dipping tube method, because of the complicating presence of ink and mineral oil. A U-tube modification of that method was used. The bottom of the osmometer tube to be calibrated was connected with a leveling bulb, and the capillarity correction was then equal to the difference between the levels of fluid in tube and bulb. It was found

that the difference when saline alone filled the U-tube was not affected by covering the saline in the tube with oil and ink columns of the usual height. When serum filled the system, the capillarity correction was much lower; but for serum covered with oil and ink, the correction was the same as that for saline covered with oil and ink. Evidently it is the surface tension of the ink which determines capillarity. The oil cap above the ink does not reach the glass in a wet clean tube; and the trace of serum presumably contaminating the ink seems to have no significant effect. A constant capillarity correction was therefore used for a given tube.

CRITICAL CONSIDERATIONS

The reliability of the method was tested in three ways. First, duplicate automatic observations were made upon various serums. Five typical pairs of such results appear in Table I (Experiments 1, 2, 3, 4, and 5). The maximum difference between them of 1.3 cm. water and the average difference of 0.7 cm. water are of the same order of magnitude as those reported by Krogh and Nakazawa (1927) for the same type of osmometer.

TABLE I

RESULTS BY THE NEW METHOD COMPARED WITH RESULTS BY THE WELLS-KROGH AND NAKAZAWA METHOD

OBSERVATION NO.	S-L METHOD	DIFFERENCE	W-K-N METHOD	ABSOLUTE DIFFERENCE	RELATIVE DIFFERENCE	TIME TO REACH EQUILIBRIUM
	CM. H_2O		CM. H_2O	CM. H_2O	%	HOURS
1	23.5	1.3	24.7	-1.2	- 4.9	19
	22.2			-2.5	-10.1	18
2	22.0	0.8	24.0	-2.0	- 8.3	12
	21.2			-2.8	-11.7	14
3	21.5	0.6	25.1	-3.6	-14.3	9
	22.1			-3.0	-12.0	11
4	6.7	0.5	7.5	-0.8	-10.7	15
	7.2			-0.3	- 4.0	18
5	35.7	0.5	36.7	-1.0	- 2.7	12
	36.2			-0.5	- 1.4	11
6	24.4		25.5	-1.1	- 4.3	19
7	24.4		25.8	-1.4	- 5.4	15
8	23.2		23.5	-0.3	- 1.2	16
9	22.1		24.3	-2.2	- 9.0	7
10	31.5		30.0	+1.5	+ 5.0	16
11	36.9		36.5	+0.4	+ 1.1	15
12	22.1		23.6	-1.5	- 6.3	18
13	20.6		22.5	-1.9	- 8.4	21
14	31.7		35.1	-3.4	- 9.7	15
15	20.5		24.5	-4.0	-16.3	18
Mean		0.7		-1.6	- 6.7	15
Extreme		1.3		+1.5	+ 5.0	7
				-4.0	-16.3	21

Second, automatic measurements have been compared with those obtained simultaneously by Wells' modification (1933) of Krogh and Nakazawa's method (1927). These experiments were conducted at room temperature (22-27° C.) with the osmometers of both types immersed in large baths of water to protect them from transient temperature fluctuations. As shown in Table I, the extreme difference in the values provided by the two methods is 4 cm. water, the mean difference 1.6 cm. water.

In all but two instances, however, the automatic apparatus yielded results slightly lower than those of the Wells technic. Such a systematic difference suggests that a constant error is present in one of the methods. The Wells method, as used by Wells, Miller and Drake (1935) to determine the osmotic pressure of hemoglobin solutions, yielded values which agreed closely with the osmotic pressure predicted by Adair's equation (Adair, 1925). It seemed possible, therefore, that the automatic recording and balancing system might introduce some constant error.

To test this possibility, the automatic apparatus was used to measure the capillarity values of the osmometer tubes, already measured by the U-tube method described above. The osmometer was assembled as usual, except that the cellophane membrane was punctured. The bottom of the lower collar was connected through a perforated stopper and rubber tubing with the bottom of a leveling bulb. From the leveling bulb through the punctured membrane to the osmometer tube, the system was filled with physiologic salt solution. When the usual columns of oil and ink had been placed above the surface of the salt solution in the osmometer tube, the top of the tube was connected to the electrolysis chamber and the recording manometer in the ordinary way. As soon as electrical connections were closed, electrolysis pushed the ink-oil interface into the light beam and held it there. Then the surface of the saline in the leveling bulb was placed level with the meniscus at the top of the fluid column in the osmometer tube. Since the two forces, P_h and B, were eliminated by these procedures, the only force opposed by electrolysis was C, the capillarity, which could therefore be read directly on the record. A steady value was quickly reached. With a syringe and a second recording manometer attached to the top of the leveling bulb, known increments of hydrostatic pressure were then added to that of capillarity, to test the reliability of the apparatus in balancing and recording a range of pressure up to 80 cm. water. The records of both manometers ran consistently parallel. For a given tube, the average value for capillarity given by the automatic method agreed to within a millimeter of water with the capillarity value obtained by the U-tube method.

This excellent agreement demonstrated that the automatic apparatus for balancing and recording pressure was accurate in an absolute as well as in a relative sense. It must be remembered, however, that when the apparatus was applied to a measurement of colloid osmotic pressure, an air bubble was present below an intact membrane in the osmometer. It seems possible that that bubble introduced some factor not completely represented by B, the bubble correction. The small but consistent difference between the colloid osmotic pressures measured simultaneously by our method and that of Wells may have been due to this, or to the unlike arrangement of membranes, or to the different lengths of time required to reach equilibrium by the two methods.

The osmometer which has been described possesses certain advantages over that of Krogh and Nakazawa (1927), after which it was patterned. Its glass walls permit thorough cleaning and make it easy to avoid air bubbles. Assembly by means of two metal collars, which remain fixed in relation to

one another and to the membrane, while screws approximate them, seems safer than an arrangement by which essential parts of the chamber turn upon one another, with danger of rumpling the washers, or of roughening their bearing surfaces.

In comparison with the chamber of Urban (1934), the glass chamber has the very important advantage of enclosing the colloidal solution within a single space. It is doubtful whether a true equilibrium can be obtained within a reasonable time when a considerable volume of the colloidal solution is recessed from the membrane. The transparency of the glass chamber is an additional advantage. On the other hand, the glass chamber supports the membrane less rigidly than does Urban's. This may be responsible for the longer time required to reach equilibrium in our experiments. Urban reports that with grade 600 cellophane, four hours sufficed to reach an equilibrium by his method, though he does not define "equilibrium."

In Urban's apparatus the rising serum level closed a mercury-platinum contact, whereas in the apparatus described in this paper the rising serum activated a photronic cell. At one stage of developing this method, platinum contacts were tried and at last rejected because, however carefully arranged, the surface of the mercury fouled after a few hours and diminished the sensitivity of control. It was for this reason that a photoelectric unit was arranged.

SUMMARY

An apparatus is described which automatically balances the colloid osmotic pressure of serum by means of a photoelectric unit and the gas pressure generated by electrolysis. It also writes a continuous kymograph record of pressure changes. The consistency and possible errors of the method are considered.

REFERENCES

Adair, G. S.: A Critical Study of the Direct Method of Measuring the Osmotic Pressure of Haemoglobin, Proc. Roy. Soc. A. **108**: 627, 1925.

Krogh, A., and Nakazawa, E.: Beiträge zur Messung des kolloidosmotischen Druckes in biologischen Flüssigkeiten, Biochem. Ztschr. **188**: 241, 1927.

Urban, F.: Automatic Recording Micro-osmometer for Colloidal Osmotic Pressures, Rev. Sc. Instr. **5**: 375, 1934.

Wells, H. S.: A Modified Krogh Osmometer for the Determination of the Osmotic Pressure of Colloids in Biological Fluids (with Directions for Its Use), J. Tenn. Acad. Sc. **8**: 102, 1933.

Wells, H. S., Miller, D. G., Jr., and Drake, B. M.: The Validity of Rapid Determinations of the Osmotic Pressure of Protein Solutions, J. Clin. Investigation **14**: 1, 1935.

NOTE ON BLOOD VOLUME DETERMINATIONS*

C. B. Weld, M.D., and H. E. Woodward, Ph.D., Toronto, Canada

DURING the course of some experimental work on dogs, we have had occasion to make a number of blood volume determinations. We have used a modification of the dye method of Keith, Rowntree and Geraghty,[1] injecting 1.0 c.c. of an approximate 1.5 per cent aqueous solution of brilliant vital red for each 5 kg. dog, and determining by a colorimeter the extent to which the dye has been diluted by the plasma of the animal. The yellow color of the plasma is compensated by making up the standard dye solution (the colorimeter standard) in plasma which has been obtained from the animal before injection of the dye. The standard is prepared by diluting 3 c.c. of the 1.5 per cent dye solution[2] to 80 c.c. with water and adding 0.1 c.c. of this to 1.0 c.c. plasma, the volume then being made up to 2.0 c.c. with water. In our experience better color matches may be obtained by this procedure than when a larger volume of more dilute dye is added to the plasma.

As is well known, one of the difficulties of the method is that a trace of hemolysis in either of the plasma specimens will seriously interfere with the colorimetric matching of the red dye. It occurred to one of us that a chemical reagent might be found which would destroy or bleach the hemoglobin without affecting the dye, and it has been found that such a reagent exists in the form of a 3 per cent solution of hydrogen peroxide. By its use, the color matching of the standard against the unknown is greatly facilitated, and a higher degree of accuracy is consequently obtained. Only a small amount of the peroxide is required, 0.1 c.c. added to the plasma and dye being quite sufficient. The total volume is, as before, made up to 2.0 c.c. The peroxide is, of course, added to both standard and unknown, and the samples are allowed to stand for about an hour before making the color comparison, as the peroxide action is slow. At the end of this time the samples are gently shaken in order to remove the retained oxygen. Gross hemolysis, if present, is readily detected and peroxide treatment in such cases is not recommended, as the increased amount of peroxide necessary may tend to affect the vital red slightly, and the produced methemoglobin is an added factor.

That the peroxide does not appreciably affect the dye except at relatively high concentrations of hydrogen peroxide over a long period of time, is shown by Table I. In these four experiments the dye (in water or plasma) was diluted to 2 c.c. before making the color comparison. The plasma and aqueous solutions contained the same amount of dye, comparisons being made against a standard containing no peroxide. The table gives the colorimeter readings, the standard being set at 10.0.

*From the Department of Physiology, University of Toronto.
Received for publication, March 25, 1936.

TABLE I

0.0 (Standard)	C.C. OF HYDROGEN PEROXIDE						
	0.0	0.05	0.1	0.2	0.3	0.5	1.0
Water + dye (1 hr.)	10.2	10.0	10.3	10.4	11.1	10.8	10.3
Water + dye (48 hr.)	10.0	10.0	10.6	11.2	12.4	12.8	About 17
Plasma + dye (1 hr.)	10.1	10.2	10.2	10.1	9.9		
Plasma + dye (12 hr.)	10.0		10.2	10.0		10.2	

Since various samples of plasma might react differently, a series of twenty samples was taken and two specimens of 1 c.c. were taken from each. To the first were added 0.1 c.c. dilute dye and 0.9 c.c. water, while to the second were added 0.1 c.c. dilute dye, 0.8 c.c. water and 0.1 c.c. hydrogen peroxide. After an hour, with the first tube of each pair as standard and with the colorimeter set at 10.0, the readings averaged 9.9 with a standard deviation of 0.4.

Since in each of these comparisons one tube contained peroxide while the other did not, comparison of color was difficult, and indeed it was impossible in two of the twenty. These two were not averaged in the above figures. The addition of hydrogen peroxide to plasma alone will usually alter somewhat the color of the plasma. That the poor color matches in these two cases was due to the effect of the peroxide on the plasma and not to a bleaching of the dye, is shown by the following observations upon them. Hydrogen peroxide was added to 1 c.c. volumes of each plasma (colored with 0.1 c.c. dilute dye) in the following amounts: 0.05, 0.1, 0.2, 0.3, 0.5, and 0.9 c.c. Volumes were in each case made up to 2.0 c.c. with water, and after one hour colorimeter readings against the first tube of each series averaged 10.1 ± 0.3. The color matches were good. The readings would, of course, have been higher if the dye were being bleached by the larger amounts of peroxide.

Several other series of experiments were made, with added amounts of aqueous hemoglobin and with varying amounts and dilutions of dye. Figures for these groups are not included as they merely confirm the above findings.

SUMMARY

The addition of small amounts of hydrogen peroxide to both the "standard" and "unknown" solutions of a blood volume determination is recommended. It will remove the confusing factor introduced by hemolysis and improve the accuracy of the readings. Evidence is presented to show that under the conditions of the method the dye itself is not affected appreciably by the peroxide.

REFERENCES

1. Keith, N. M., Rowntree, L. Y., and Geraghty, J. T.: A Method for the Determination of Plasma and Blood Volume, Arch. Int. Med. **16**: 547, 1915.
2. Smith, H. P.: Fate of Intravenously Injected Dye (Brilliant Vital Red) With Special Reference to Its Use in Blood Volume Determination, Bull. Johns Hopkins Hosp. **36**: 325, 1925.

AN IMPROVED TECHNIC FOR THE DETERMINATION OF INSENSIBLE PERSPIRATION*

RALPH F. NICKERSON, PH.D., WORCESTER, MASS.

A DECADE ago Benedict and Root[1] reviewed the literature on insensible perspiration and, as a result of their own work, pointed out that there exists in man an extremely high correlation between the oxygen consumption rate and the rate of weight loss due to insensible perspiration. Later work, especially by Benedict and Wardlaw[2] and by Wilson,[5] has indicated the practicability of the method also. Under suitably controlled conditions the rate of weight loss can be used as an accurate index of the basal metabolic rate. The defects of the standard basal metabolic rate methods for children are well known and Hoskins[4] has stressed the need for more adequate methods for psychotics.

Three advantages of the weight-loss method may be emphasized. The first is its simplicity. The untrained subject neither has attached to him a complex machine with an attendant disturbance of his equanimity, nor does he have his nose forcibly occluded with the suggestion of suffocation. He simply lies down on a suspended couch and rests. By suggestion the unmolested repose is quietly conducive to basality and to the release of psychomotor tension. A second advantage is that the method does not depend on respiratory tracings. The irregular respiration so common to neurotic and psychotic patients makes little, if any, difference in the rate of weight loss. Finally, the weight-loss method yields accurate, continuous determinations over long or short periods without the introduction of fatigue or habituation factors.

Work at the Worcester State Hospital and elsewhere has shown that the basal metabolism of the schizophrenic patient is indubitably anomalous.[3] Study of the anomaly at this hospital has been rendered difficult, and sometimes impossible, by the irregular respirometer tracings, by the lack of cooperation from the subjects and by the intrusion of indeterminate degrees of excitement which displaces the basal rate. For these reasons the insensible perspiration method, with its promise of more reliable data, has been adopted.

A Sauter balance of 100 kg. capacity was mounted on a rigid support. One scale of the balance is a caned, rectangular framework sufficiently large to bear a reclining man. The cane and wood of this light structure were thoroughly varnished to preclude moisture exchanges with the surroundings. Sling chains from the corners attach this bed to the knife-edge yoke. The other scale is fashioned to carry the heavy counterbalancing lead weights.

*From the Research Service of the Worcester State Hospital.
Received for publication, February 19, 1936.

Certain technical difficulties at first interfered with the use of the apparatus. Hitherto, rates of weight loss were determined with this balance by placing the subject upon the bed and almost counterbalancing his mass with weights on the opposite scale. The balance was then set in motion. Since the subject was continually losing weight, a point of balance, which was indicated by equal excursions of the pointer from the zero point, was soon reached. A 10-gram increment was placed on the scale with the subject and the lapse of time before the apparatus reached a new point of balance was noted. Obviously, this technic not only prevented the study of weight changes during lesser periods of time but also required the constant attention of an operator. Moreover, the very delicacy of balance was a hindrance. An inadvertent movement on the part of the subject would start in the apparatus a swinging which would persist for a matter of minutes and seriously interfere with the determination.

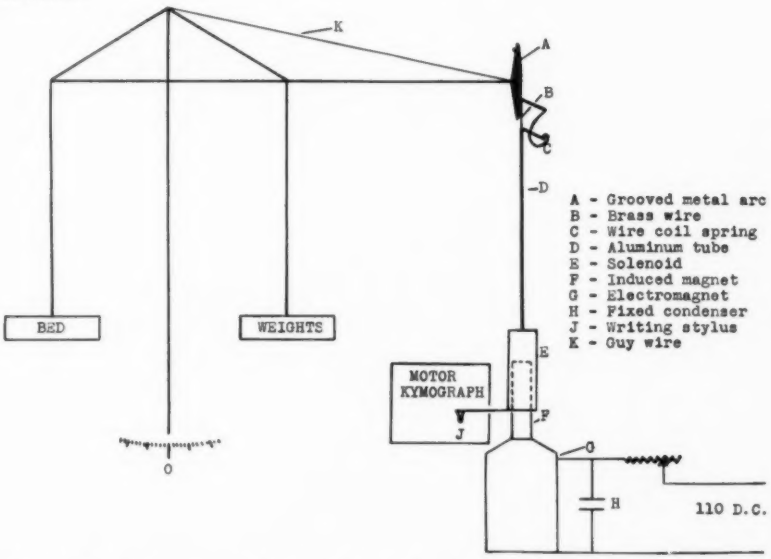

Fig. 1.—A sketch of the structural features of the recording and damping apparatus.

This paper is a report on the methods that were evolved to control these experimental difficulties. Above all, the objects were to achieve a method of arresting the too easily initiated, periodic oscillations and one of producing continuous readings of the weight loss. A subsidiary problem was to obtain graphic records without the introduction of friction which would detract from the maximum sensitivity of the apparatus. Photometer methods were rejected because such extreme caution must be observed in the handling of light-sensitive materials. Hence, the problem resolved itself, first, to the adaptation of a writing stylus to a state of minimum recording friction.

The final arrangement of the apparatus is delineated in Fig. 1.

The balance beam was extended horizontally by means of a steel rod and a guy wire so that the distance from the center knife edge to the end of the extension was equivalent to the distance from the knife edge to the end of the zero pointer. The end of the extension then executed an arc which cor-

responded identically to the arc traversed simultaneously by the zero pointer and possessed, therefore, the same inherent sensitivity as the pointer. The writing point and ink cup unit was suspended by a fine steel wire from an aluminum tube attached as shown. Lateral twisting of the pen was overcome with a delicate coil spring which prevented the stylus from moving away from the kymograph paper. Since the stylus was displaced only slightly from its position of rest as a pendulum by contact with the paper, the total friction of the writing system was negligible in comparison with that of the balance and its air resistance. In fact records were made which show that oscillations due to a major impulse did not subside for eight minutes (Fig. 2). The oscillatory record shown is proof that the balance is practically free from frictional damping.

The problem of damping* these oscillations was solved by an adaptation of the principle of Lenz. A strong electromagnet was constructed and placed below the end of the beam extension. A right cylinder of steel shafting of

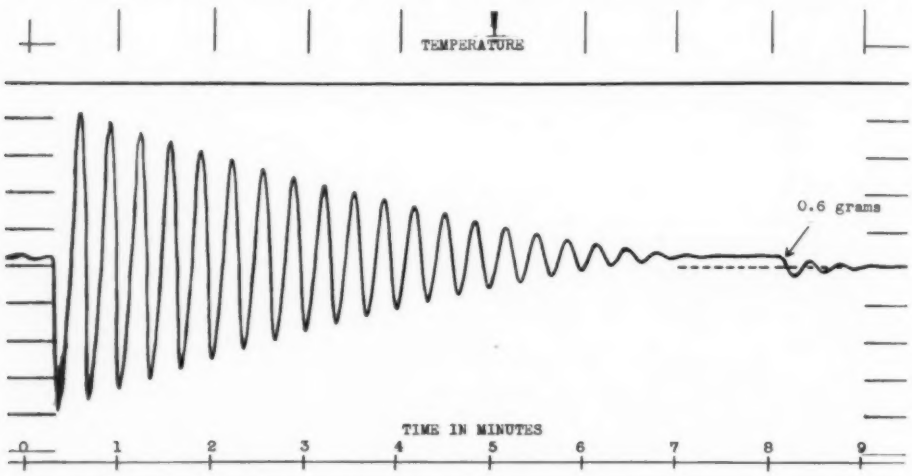

Fig. 2.—Record of the oscillatory response of the balance to a major impulse. The effect of changing the load by one lead shot is shown also.

inch diameter and ten-inch length was set vertically on the pole piece of the magnet. A short-circuited solenoid of copper wire was suspended from the beam extension so that it moved up and down around the induced magnet without lateral contact. This device added a small weight but no friction to the balance system. As this solenoid *moves* in the field of the fixed magnet, it acquires a magnetic field which in turn always opposes the field of the fixed magnet. The interaction of these opposing magnetic fields tends to keep the solenoid stationary. Thus, the vertical component of every motion of the balance system is opposed and the balance is inclined to stay at rest without any loss of its sensitivity. With this arrangement a single excursion of the beam is sufficient to destroy all effects of a major impulse in an elapsed time of about twenty seconds. In other words, the balance has been converted to a "deadbeat" instrument. This action is shown in Fig. 3.

*The author is not unaware that damping devices of various kinds are available commercially, but, to his knowledge, none meets the stringent requirements of this problem.

The response to a sustained weight change under these conditions is always slightly slower than the undamped response of the instrument due to the drag of the magnetic fields but the magnitude of the response is not lessened. The proof of this statement is given by a comparison of the responses to a 0.6 gram weight change by the undamped system (Fig. 2) and the damped system (Fig. 3). In each case the displacement is 3.5 mm., or about 5.8 mm. per gm.

The variable resistance shown in Fig. 1 is used to adjust the magnet current in order that the instrument can be made "deadbeat" with different loads. The condenser acts as a current stabilizer.

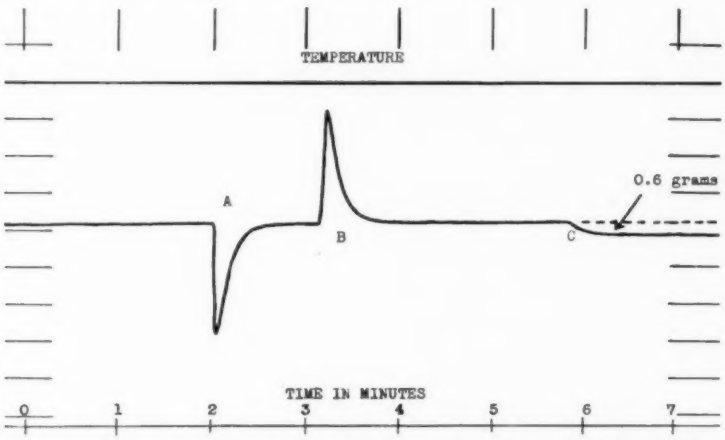

Fig. 3.—Major impulses (*A* and *B*) electromagnetically damped. The response of the damped balance to a load change of one lead shot is shown also (*C*).

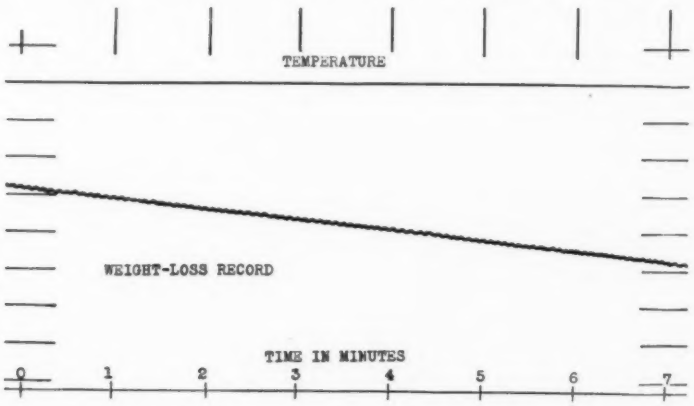

Fig. 4.—A seven-minute record on a resting subject.

A portion of an actual weight-change record of a 145-pound man is shown in Fig. 4. The respiration may be seen superimposed upon the weight-loss record. The seven-minute weight change is 4 gm., a rate of 34.0 gm. per hour.

For continuous, graphic records it is necessary to remove 10 gm. weights from the counterbalancing scale from time to time in order to keep the balance in the region of its zero. A staggered line results from which rates are easily calculated. A 10 gm. weight difference causes the beam of the balance to move through an angle of 2.4°. Tests have shown that the displacement of the

balance is a linear function of the weight change over this entire interval. For this reason a point of absolute balance does not have to be determined at all. The factor for the conversion of graphic displacements to grams can be calculated from the vertical length effected on the record by a 10 gm. decrement.

Weight changes can be estimated on the kymographic record to about half a millimeter. This corresponds to about 0.10 gm., or roughly to 1 part in 800,000 for an average-sized man.

A simultaneous record of the room temperature is registered on the kymograph by means of a metal linear expansion thermometer. An 800 cm. length of No. 28 B and S gauge constantin wire was attached to the opposite wall of the room and brought across over a pulley to the kymograph. The wire was fastened near the pivoted end of an arm about 20 cm. long. The writing stylus at the other end of this arm yielded a 14 to 1 magnification of the thermal contraction and expansion of the wire or 0.23 cm. vertical variation per degree centigrade. The advantage of this thermometer is its almost instantaneous response which originates in its extremely low heat capacity.

A timing device completed the apparatus. The timer consists of a synchronous telechron motor which, once each minute, activates the magnetic relay to which a writing stylus is attached. The time line that results serves also as a base line for measurements on the final record.

SUMMARY

A method is described for the graphic recording of weight losses due to the insensible perspiration. The outstanding features of the apparatus are an electromagnetic damping arrangement and a writing stylus on a torsion pendulum. The damping device, which does not affect the sensitivity of the balance, makes continuous weight-loss records feasible and is a decided improvement upon the previous methods. The torsion writing system is practically frictionless. The advantages of insensible perspiration for the estimation of the basal metabolic rates of untrained subjects, children, neurotics, and psychotics are discussed.

REFERENCES

1. Benedict, F. G., and Root, H. F.: Insensible Perspiration: Its Relation to Human Physiology and Pathology, Arch. Int. Med. **38**: 1, 1926.
2. Benedict, F. G., and Wardlaw, H. S. H.: Some Factors Determining the Insensible Perspiration of Man, Arch. Int. Med. **49**: 1019, 1932.
3. Devine, H.: Recent Advances in Psychiatry, ed. 2, Philadelphia, 1933, P. Blakiston's Son & Co., p. 216.
4. Hoskins, R. G.: Oxygen Consumption (Basal Metabolic Rate) in Schizophrenia, Arch. Neurol. & Psychiat. **28**: 1346, 1932.
5. Wilson, G. E.: Insensible Perspiration: Its Relation to Basal Metabolism, Arch. Int. Med. **52**: 378, 1933.

STATISTICAL METHODS FOR THE LABORATORY AND CLINICAL INVESTIGATOR*

The Chi-Square Test of Homogeneity

Paul D. Rosahn, M.D., New Haven, Conn.

> "Some people hate the very name of statistics, but I find them full of beauty and interest. Whenever they are not brutalized, but delicately handled by the higher methods, and are warily interpreted, their power of dealing with complicated phenomena is extraordinary. They are the only tools by which an opening can be cut through the formidable thicket of difficulties that bars the path of those who pursue the Science of Man."†
>
> —Sir Francis Galton.

THE attitude toward statistics which Galton deprecated some fifty years ago is today expressed in the scoffing statement, "You can prove anything by statistics." One might say with equal merit, "You can prove anything by chemical analysis." It is important to remember that the statistical method is a scientific instrument in much the same sense that the microscope and the colorimeter are scientific instruments. Both depend for their value in interpreting biologic phenomena on the astuteness, judgment and experience of the observer. An inexperienced observer who sees a section of a suspect tissue under the microscope may call it benign when in reality it is malignant, but one would never think in this instance of blaming the microscope for the incorrect designation, since the observer alone is at fault. Similarly, when statistics appear to lead to erroneous conclusions—"there are liars, damn liars and statistics"—the capacity of the interpreter or compiler to deal with his material should be questioned, and not the statistical method itself. To carry the analogy further, the trained histopathologist by employing that staining technic which will best aid in the identification of a particular structure will have no difficulty in locating it; the novice, on the other hand, failing to utilize the proper methods, will report the absence of the phenomenon under investigation. Here again, the staining technic is not at fault, but rather the individual upon whom rests the choice of several procedures which vary in their capacity to delineate.

To the clinical investigator and laboratory worker who constantly deal with variables affected by a multiplicity of factors, a knowledge and understanding of certain fundamental statistical procedures is invaluable. Contrary to the usual impression, it is not necessary to understand the higher mathematics in order to utilize the statistical method intelligently. Anyone with a working

*From the Research Division, Public Health Institute, Chicago, Ill.
Received for publication, March 31, 1936.
†*Natural Inheritance*, Macmillan and Co., London and New York, 1889, p. 62.

knowledge of algebra can apply the comparatively simple formulas which have been derived by specialists in the science. The student will of course wish to comprehend the mathematical steps by which these formulas were developed, but this information is not essential. Few people who drive a car understand the principle and construction of the gasoline motor.

The application of statistical methods to quantitative data enables the investigator to describe a population succinctly and precisely, so that the limits of the variability of that population are incorporated in his description of it; to determine whether variability in one set of phenomena is related to variability in another, and if this relationship exists, to describe its direction; to ascertain whether one group of observations on a variable is similar to or different from another group of observations on the same variable, or otherwise stated, whether both groups are samples from the same or different populations of data. Numerous variations of these problems arise in the analysis of any particular set of data, and most of them can be resolved by the application of statistical methods now in use.

The investigator in reviewing his experimental data is often confronted with the problem of deciding whether or not the differing frequencies of an attribute in two or more groups are significant. A common procedure in appraising the significance of a difference is to be guided by its magnitude—a large difference is significant, a small difference insignificant. This procedure frequently leads to erroneous conclusions, for very often large differences can be shown to be lacking in significance, and small differences to be highly significant. It is the purpose of this paper to discuss this type of problem and to describe a simple statistical method which has been found useful in analyzing such data. The problem is related to the concept of homogeneity, and the method is the Chi-square (χ^2) test of homogeneity.*

Homogeneity and Heterogeneity.—The simplest example of homogeneity is that of one variable in a given population. A group composed entirely of males is homogeneous with respect to the variable sex, while another group composed of males and females is heterogeneous as regards sex. It is evident that a group may be both homogeneous and heterogeneous, depending on the attribute under consideration. Thus a group of men (homogeneous as regards sex), if composed of individuals of different racial origin, would be heterogeneous with respect to the variable race. A more complicated situation arises however when the frequency of an attribute in more than one group is considered. Suppose we are considering the attribute "eye color" in two samples A and B, and we find that the frequency of blue-eyed individuals is 30 per cent in each group. We are justified in concluding that these two groups are homogeneous as regards the incidence of blue-eyed individuals, that is, they are both samples from the same population in which the incidence of blue-eyed individuals is 30 per cent. If however the frequency of blue-eyed individuals in sample A is 10 per cent, and in sample B is 40 per cent, it becomes important to know whether the differing frequencies in these two samples are due to the fact that the original populations from which they were drawn are

*This test has recently been applied to geological data. See Eisenhart, C.: A Test for the Significance of Lithological Variations, J. Sedimentary Petrology 5: 137, 1935.

actually different, that is, are heterogeneous, or whether the difference is the result of random sampling from the same population. For even if two samples are known to have been selected from the same population they may differ with regard to the frequency of an attribute, such difference resulting from fortuitous circumstances in selecting the two samples. It becomes evident therefore that before one can determine whether two or more samples are statistically homogeneous (have been selected from the same parent population) or statistically heterogeneous (have been selected from different parent populations) one must evaluate the extent of the influence of random sampling on the observed differences. The following example is illustrative:

Suppose that a random selection of 410 individuals is composed of 190 males and 220 females, while a second group of 590 comprises 210 males and 380 females. The proportion of the sexes in these two samples is actually different, the first group containing 46.3 per cent males and the second, 35.6 per cent. If this difference can be accounted for by the chance error of sampling, then the two groups have very likely been drawn from the same population in which the proportion of the sexes approaches that found in the samples. If, however, the difference cannot be ascribed to the error of random selection, then the two samples are heterogeneous, having been drawn from two different populations, with differing proportions of the sexes.

The Chi-square (χ^2) test of homogeneity is admirably adapted to assist in selecting one or the other of the above suppositions as the more probable. The text of Fisher[1] has been freely drawn on for the following explanatory remarks, and the interested reader is referred to this excellent monograph for further details. Our problem can best be shown in what is called a 2×2 or four-square table (Table I).

TABLE I

THE FREQUENCY OF MALES AND FEMALES IN TWO GROUPS. OBSERVED VALUES

SEX	GROUP I	GROUP II	TOTAL
Male	(a) 190	(b) 210	(a + b) 400 (40%)
Female	(c) 220	(d) 380	(c + d) 600 (60%)
Total	(a + c) 410	(b + d) 590	(a + b + c + d) 1000

These two groups contain 1,000 individuals, 400 or 40 per cent of whom are males, and 600 or 60 per cent of whom are females; this is the proportion of males and females which we should expect to find in any sample taken from this total population of 1,000 individuals. Were the two groups under consideration ideal random samples from this population, Group I would contain 164 males (410×40 per cent) and 246 females (410×60 per cent) and Group II would consist of 236 males (590×40 per cent) and 354 females (590×60 per cent). Actually, however, Group I contains 190 males and 220 females, and Group II, 210 males and 380 females. The frequencies which we expect to find in the two groups, if they were random samples from a population containing 40 per cent males and 60 per cent females, are best shown in a second table (Table II).

TABLE II

THE FREQUENCY OF MALES AND FEMALES IN TWO GROUPS. EXPECTED VALUES

SEX	GROUP I	GROUP II	TOTAL
Male	(a) 164	(b) 236)	(a + b) 400 (40%)
Female	(c) 246	(d) 354)	(c + d) 600 (60%)
Total	(a + c) 410	(b + d) 590)	(a + b + c + d) 1000

By definition χ^2 is taken to equal

$$S \frac{\chi^2}{m}$$

where S is the sum of the values for each class, x is the difference between the observed and the expected frequency, and m is the expected frequency. It will be noted that the greater the difference between the observed and expected values, the larger will be the value for χ^2, and thus the degree of divergence from expectation is represented by the magnitude of χ^2. In the present example x is equal to 26 (in box a, observed frequency of 190 differs from expected frequency of 164 by 26; similarly for box b, c, and d), and x^2 is 676. The following arithmetical processes give the total S or χ^2:

$$\text{Box a. } \frac{\chi^2}{m} = \frac{676}{164} = 4.12$$

$$\text{Box b. } \frac{\chi^2}{m} = \frac{676}{236} = 2.86$$

$$\text{Box c. } \frac{\chi^2}{m} = \frac{676}{246} = 2.75$$

$$\text{Box d. } \frac{\chi^2}{m} = \frac{676}{354} = 1.91$$

$$S = 11.64 = \chi^2$$

Degrees of Freedom.—In order to interpret this value of χ^2, two explanatory statements are necessary. The first is concerned with the concept "degrees of freedom." It will be observed that if the expected value for any box is known, the expected value for all the boxes can be ascertained by subtraction from the marginal totals. Thus if we have calculated the expected value, 164, for box (a), as described above, the expected value for box (b) can be found by subtracting this frequency from the total of 400. Similarly the expected value for box (c) can be ascertained by subtracting 164 from the total 410, and of box (d) by subtracting 236 from the total 590. The number of degrees of freedom *n*, is the number of expected values which have to be determined in order to find all the expected values. Since in the present example by finding the expected value for any box all the expected values can be determined from the marginal totals, $n = 1$. It is evident that $n = 1$ for all four-square tables. The following general rule will enable one to determine the number of degrees of freedom in any problem: if y is the number of groups and z the number of categories, the number of degrees of freedom is represented by the formula $(y-1)(z-1)$.

Interpretation of Significance.—The second explanatory statement is concerned with the interpretation of significance. This will depend to some extent on the judgment of the investigator, and the nature of the special problem, but statisticians have accepted certain criteria as standards. In general, when the probability of a difference being due to chance or random sampling alone is one or less than one in 100 ($P = 0.01$ or 0.01–), the differ-

ence is highly significant, and the populations presenting such a difference are accepted as being statistically heterogeneous. To illustrate, let us assume that the difference between two samples is represented by P = 0.01. This indicates that if we were to take 100 pairs of such samples from a known population, we should expect only one pair out of the hundred to show a difference equal to or greater than that actually observed between the original two samples. In a statistical sense the two samples are significantly heterogeneous. When the chances that an observed difference occurred through random sampling alone are between one and five in 100 (P = 0.01 to 0.05), the difference is usually considered to be probably significant, and the two populations probably heterogeneous. In this case the accepted rigid criterion for heterogeneity has not been satisfied, but it is highly probable that the addition of further data to each group will assist in demonstrating significant heterogeneity, i.e., will bring the probability of chance occurrence down to one in 100 (P = 0.01). When the values for two populations of data can be expected to occur through random sampling in more than five trials in 100 (P = 0.05+), significant heterogeneity between the two populations has not been demonstrated. The failure to demonstrate heterogeneity is not to be interpreted as indicating homogeneity. If the purpose is to show positive homogeneity between two samples, P should be not too far from the neighborhood of 0.5, that is, the probability of similar events occurring by chance alone should be around 50 in 100 trials.

To return to the example cited in Tables I and II, $\chi^2 = 11.64$ and the number of degrees of freedom = 1. Tables are available, particularly those published by Fisher (pages 110, 111), for translating various levels of χ^2 for different degrees of freedom into terms of probability. These tables, which are based on homogeneous populations of data, indicate the relative probability of obtaining by random selection alone a value for χ^2 equal to or greater than that actually observed. For one degree of freedom, when χ^2 is less than 3.841 the probability that the difference noted occurred by chance is more than five in 100 (P = 0.05+). If $\chi^2 = 6.635$ or more, the probability that the difference was due to random sampling alone is one or less than one in 100 (P = 0.01 or 0.01–) and the populations are statistically heterogeneous. When $\chi^2 = 11.64$, and there is one degree of freedom, as is the case in the present instance, P = 0.01–, and the populations are thus highly significantly different. We may interpret the result as indicating that Groups I and II are significantly heterogeneous as regards the proportion of male and female components, or that the frequency of males in Group I is significantly greater, and of females significantly fewer than the frequencies in Group II, or that there are significantly more males in Group I and significantly fewer males in Group II than would be expected from random sampling from the same population.

The example cited illustrates the importance of sufficiently large samples in order to demonstrate heterogeneity. In the following four-square table (Table III) the proportion of males to females in the two groups and in the totals is practically the same as that postulated in the previous example (Table I), and yet the value for χ^2 indicates that heterogeneity is absent.

TABLE III

THE FREQUENCY OF MALES AND FEMALES IN TWO GROUPS. OBSERVED AND EXPECTED VALUES

SEX	VALUE	GROUP I	GROUP II	TOTAL
Male	Observed	(a)	(b)	46 (40.35%)
	Expected	24.0	22.0	46 (40.35%)
		21.0	25.0	
Female	Observed	(c)	(d)	68 (59.65%)
	Expected	28.0	40.0	68 (59.65%)
		31.0	37.0	
Total	Observed	52.0	62.0	114
	Expected	52.0	62.0	114

The following calculations show the derivation of χ^2:

$\chi = 24 - (52 \times 40.35\%)$ or $24 - 21.0 = 3.0$

$\chi^2 = 9.00$

Box a. $\frac{\chi^2}{m} = \frac{9.00}{21.00} = 0.43$

Box b. $\frac{\chi^2}{m} = \frac{9.00}{25.00} = 0.36$

Box c. $\frac{\chi^2}{m} = \frac{9.00}{31.00} = 0.29$

Box d. $\frac{\chi^2}{m} = \frac{9.00}{37.00} = 0.24$

$S = 1.32 = \chi^2$; $n = 1$, $P = 0.25$

The probability that the distribution shown in Table III might occur by chance in sampling from a homogeneous population is about 25 in 100, and following the criteria discussed under the interpretation of significance, these two samples cannot be said to be significantly heterogeneous. Since these groups are rather small, one would suspect that larger samples might prove them to be representative of different populations. This was actually the case when larger samples were considered, as shown in Table I. It becomes evident that in order to interpret such data, actual frequencies should be cited rather than percentages, for the latter give no indication of the size of the data on which they are based. Moreover, since the error of random sampling decreases as the size of the sample is increased, great care should be exercised in drawing conclusions from small samples.

Another example of the use of χ^2 for testing homogeneity is the following: Kemp and Menninger[2] studied the influence of the treatment of early syphilis on the incidence of neurosyphilis, and based their findings on a group of 680

TABLE IV

THE INCIDENCE OF NEUROSYPHILIS IN UNTREATED MALES AND FEMALES. OBSERVED AND EXPECTED VALUES (ADAPTED FROM KEMP AND MENNINGER[2])

SEX	VALUE	NEUROSYPHILIS	NO NEUROSYPHILIS	TOTAL
Male	Observed	(a)	(b)	125 (49.4%)
	Expected	71	54	125 (49.4%)
		67	58	
Female	Observed	(c)	(d)	128 (50.6%)
	Expected	64	64	128 (50.6%)
		68	60	
Total	Observed	135	118	253
	Expected	135	118	253

patients of whom 330 were males and 350 were females. Thirty-eight per cent of the males and 36.5 per cent of the females had never received any treatment, and of this untreated group, 57.0 per cent of the males developed neurosyphilis as contrasted with 50.0 per cent of the females. Is there any difference between the sexes with regard to the incidence of neurosyphilis? Translating these percentage terms into actual frequencies the following four-square table (Table IV) is constructed.

The calculations are as follows:

$$\chi = 71 - (135 \times 49.4\%) \text{ or } 71 - 67 = 4$$
$$\chi^2 = 16$$
$$\text{Box a. } \frac{\chi^2}{m} = \frac{16}{67} = 0.24$$
$$\text{Box b. } \frac{\chi^2}{m} = \frac{16}{58} = 0.28$$
$$\text{Box c. } \frac{\chi^2}{m} = \frac{16}{68} = 0.24$$
$$\text{Box d. } \frac{\chi^2}{m} = \frac{16}{60} = 0.27$$
$$\chi^2 \text{ or } S = 1.03,\ n = 1,\ P = 0.32$$

Fisher's table indicates that for one degree of freedom, where $\chi^2 = 1.03$, P is equal to 0.32; in other words, there were 32 out of a hundred chances that the differing frequencies shown in Table IV could occur as a result of random sampling from a homogeneous population. The two populations of males and females are therefore lacking in demonstrable heterogeneity, and the conclusion is that so far as the given material is concerned, untreated syphilitic males do not differ from untreated syphilitic females with respect to the incidence of neurosyphilis.

The χ^2 test can also be employed for testing homogeneity when there are more than two classifications or samples as in the following instance: In connection with studies on a lethal dwarf mutation in the rabbit a comparison was made of the birth weights of individuals in litters containing unusually small, delicately formed living but nonviable young derived from known transmitters of the abnormality, with the birth weights of young in litters derived from normal hybrid stock.[3] In all cases routine weight determinations were employed, and the weight of each animal was expressed in terms of a percentage of that of his heaviest litter mate. The resulting percentage values were grouped in classes in descending order of magnitude, and the frequencies in each class are shown in Table V.

The expected value for any box in the normal group is obtained by multiplying the class total by 53.7 per cent, the frequency of individuals from normal litters represented in the total population of 1,053. Similarly the expected value for any box in the dwarf group is obtained by multiplying the class total by 46.3 per cent, which is the frequency of rabbits from dwarf litters in the total population. However, it will be seen that if the expected values for boxes (a), (b), (c), (d), (e), (f), and (g) have been calculated, all the other expected values can be found by subtraction from the marginal totals. The number of degrees of freedom is thus 7, or substituting in the

formula (y–1) (z–1), (7) (1) = 7. The two groups of normal and dwarf litters are very highly significantly heterogeneous, χ^2 having a far greater value than its lower limit when P = 0.01. It is evident also that the principal discrepancy between the two groups occurs in the weight classes 50.0 – 40.1 per cent, 40.0 – 30.1 per cent, and 30.0 – 20.1 per cent, where S $\frac{\chi^2}{m}$ = 40.18, 90.68, and 19.58, respectively. These three classifications alone contribute 150.44 or 84 per cent of the total χ^2 value of 177.23.

Eliminating these three classes from consideration, and applying the χ^2 test to the frequencies in the five classes from 100 – 90.1 per cent to 60.0 – 50.1 per cent, we obtain a value for χ^2 of 3.42. We find from Fisher's tables that

TABLE V

FREQUENCY DISTRIBUTION OF BIRTH WEIGHTS OF RABBITS IN NORMAL AND DWARF LITTERS.[3] WEIGHT OF EACH INDIVIDUAL EXPRESSED AS A PERCENTAGE OF THE WEIGHT OF THE HEAVIEST LITTER MATE. OBSERVED AND EXPECTED VALUES

GROUP	VALUE	100.0-90.1%	90.0-80.1%	80.0-70.1%	70.0-60.1%	60.0-50.1%	50.0-40.1%	40.0-30.1%	30.0-20.1%	TOTAL
		(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	
Normal	Observed	283.0	154.0	87.0	27.0	10.0	3.0	1.0	0.0	565
	Expected	253.5	135.3	69.3	20.9	9.1	24.2	44.0	9.1	(53.7%)
		(k)	(l)	(m)	(n)	(o)	(p)	(q)	(r)	
Dwarf	Observed	189.0	98.0	42.0	12.0	7.0	42.0	81.0	17.0	488
	Expected	218.5	116.7	59.7	18.1	7.9	20.8	38.0	7.9	(46.3%)
Total	Observed	472.0	252.0	129.0	39.0	17.0	45.0	82.0	17.0	1053
	Expected	472.0	252.0	129.0	39.0	17.0	45.0	82.0	17.0	
χ		29.5	18.7	17.7	6.1	0.9	21.2	43.0	9.1	
χ^2		870.3	349.7	313.3	37.2	0.8	449.4	184.9	82.8	
Normal	$\frac{\chi^2}{m}$	3.43	2.58	4.52	1.78	0.09	18.57	42.02	9.10	82.09
Dwarf	$\frac{\chi^2}{m}$	3.98	3.00	5.25	2.06	0.10	21.61	48.66	10.48	95.14
S		7.41	5.58	9.77	3.84	0.19	40.18	90.68	19.58	177.23

χ^2=177.23, n=7, p=0.01 when χ^2=18.48.

where n = 4 and χ^2 is 3.42 as in the present instance, then P = 0.50. In other words, if we were to take a hundred such pairs of samples from a known homogeneous population, we should find that chance fluctuations alone would produce, in 50 trials out of the hundred, differences equal to or greater than those observed. These two samples are, therefore, statistically homogeneous, indicating that the factors influencing variability between the extremes of 100.0 – 90.1 per cent and 60.0 – 50.1 per cent are operative with equal effectiveness on both groups.

It will be appreciated that the χ^2 test described herein is to be used only in problems involving the frequency of an attribute. Other methods are employed to determine heterogeneity or homogeneity between groups with respect to quantitative measurements. However, quantitative data can usually be translated into terms of frequency, as was done with the material shown in Table V, and in this form the data can be tested by the method of χ^2.

SUMMARY

The χ^2 test is a simple method for determining whether or not two or more samples of data are statistically heterogeneous as regards the frequencies of stated attributes. The test is described, and its application to several problems is illustrated.

REFERENCES

1. Fisher, R. A.: Statistical Methods for Research Workers, ed. 5, London, 1934, Oliver & Boyd.
2. Kemp, J. E., and Menninger, W. C.: The Influence of Inadequate Treatment of Early Syphilis on the Incidence and Incubation Period of Neurosyphilis, Bull. Johns Hopkins Hosp. **58**: 24, 1936.
3. Rosahn, P. D., and Greene, H. S. N.: Birth Weight Criterion of Dwarfism in the Rabbit, Proc. Soc. Exper. Biol. & Med. **32**: 1580, 1935.

310 Cedar Street

DETECTION OF HYPERPROTEINEMIA DUE TO MULTIPLE MYELOMA BY MEANS OF THE TAKATA-ARA REACTION*

Harold Jeghers, M.D., Boston, Mass.

IN SHARP contrast to the voluminous literature concerning hypoproteinemia is the paucity of data available on hyperproteinemia. Gros,[1] Wintrobe and Buell,[2] Cantarow,[3] Reimann,[4] Foord,[5] Sweigert,[6] and others have recently reported cases of hyperproteinemia associated with multiple myeloma. Foord,[5] in reviewing the literature on multiple myeloma, found less than twenty instances with high blood protein values, and pointed out that while hyperproteinemia is seen mainly in multiple myeloma, it is also found in severe dehydration, Kala-Azar, schistosomiasis and rarely in a few other poorly defined conditions. However, in multiple myeloma, the figures are usually much higher than in the other diseases mentioned. In this country, ruling out dehydration (which gives only a moderately high total protein but with a normal albumin-globulin ratio), a true hyperproteinemia can be considered strong presumptive evidence of multiple myeloma. The few available reports on hyperproteinemia are readily explained by Sweigert's[6] statement that in only 35 of 500 reported instances of myeloma were adequate quantitative investigations of plasma proteins completed.

Now that the significance of hyperproteinemia is appreciated, a more consistent search will undoubtedly be made for it. The determination of total blood proteins and the albumin-globulin ratio is a fairly difficult procedure technically, and the time consumption required will prevent this test from being used routinely in searching for instances of high total protein.

It is the purpose of this paper to point out that the Takata-Ara reaction, with particular attention paid to the amount of precipitate, can be used to

*From the Evans Memorial of the Massachusetts Memorial Hospitals; Fifth (Boston University) Medical Service, Boston City Hospital; and the Department of Medicine, Boston University School of Medicine.

Received for publication, March 21, 1936.

detect the presence of hyperproteinemia due to multiple myeloma. It requires less effort to perform than a routine urinalysis, and no expensive reagents or apparatus.

This test, originally introduced by Takata[7] to differentiate between lobar pneumonia and bronchopneumonia, was later used by Takata and Ara[8] to detect syphilitic meningitis by examination of spinal fluid. Staub[9] and Jezler[10] in 1929 modified the test to study liver function and their successful studies have resulted in the accumulation of an enormous foreign literature on the value of the Takata-Ara reaction. For a more detailed discussion on the use of the Takata-Ara reaction, papers by Tannenholz,[11] Crane,[12] Heath,[13] Ragins,[14] and Magath[15] can be consulted. It is now well established that the greatest value of the test has been in detecting advanced cirrhosis and severe degrees of hepatitis.

Gros,[1] in studying two cases of multiple myeloma with hyperproteinemia, found a positive Takata-Ara reaction in each case, and after a careful study of liver function decided the positive reaction was due to the high protein. A postmortem examination in one of his cases revealed multiple myeloma, a normal liver and absence of any other disease process which has been reported to give a positive Takata-Ara reaction. The use of the Takata-Ara reaction in detecting hyperproteinemia has not been mentioned in the American literature.

Since few references are available in English, the technic will be briefly outlined. The simplified technic suggested by Crane[12] and used with success by Heath[13] was followed.

Add 1.0 c.c. of 0.9 per cent NaCl to each of six test tubes (size of a Wassermann tube). To the first tube add 1.0 c.c. of blood serum and mix. Remove 1.0 c.c. of the mixture from the first tube and add to the second tube. After mixing the contents of the second tube, remove 1.0 c.c. of the contents and add to the third. Continue this process for each of the remaining tubes. After mixing the contents of the sixth tube discard 1.0 c.c. of the mixture. The dilutions of serum are 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. To each tube add 0.25 c.c. of a 10 per cent solution of sodium carbonate. Mix each tube well. To each tube, now add 0.15 c.c. of 0.5 per cent mercuric chloride solution and again mix each tube. Thorough mixing after the addition of each reagent is essential to prevent the appearance of a reddish granular precipitate due to reaction between the reagents used. A good grade of distilled water should be used to prepare the solutions.

Allow the six tubes to stand at room temperature for eighteen to twenty-four hours. A positive reaction is present when a definite pearly flocculent precipitate persists in any tube after eighteen hours. Occasionally at the start of the test a fine white granular precipitate appears, only to disappear within a half hour. This is without significance. The average positive test in cirrhosis and severe hepatitis is a well-marked, pearly flocculent precipitate in the third, fourth, and fifth tubes and occasionally to a lesser degree in the other tubes. In contrast to this, an unusually heavy precipitate, which was often present in all tubes, formed in the hyperproteinemia cases, and the height of the precipitate was two or more times that seen in the liver cases.

In this paper no attempt is made to present the clinical details of these cases, but interest is centered on the laboratory data which are summarized in Table I.

In collecting data during the past year on the Takata-Ara reaction, many tests were done routinely in various types of clinical cases that are found on

a general medical service. In Case 1 a degree of precipitation at least two or more times as heavy as was commonly seen in the positive tests, was noticed. To one accustomed to seeing the usual degree of precipitation, this phenomenon was quite impressive and led to the determination of blood proteins. The total protein was 12.4 gm. per 100 c.c., globulin 8.9 gm. per 100 c.c., and albumin 3.5 gm. per 100 c.c. Such high values at once suggested the diagnosis. These results (both Takata-Ara reaction and protein values) were checked several times over a period of several months with similar results. Liver function tests in this case were normal. At postmortem examination the clinical diagnosis of multiple myeloma of the plasma cell type was verified. Both grossly and microscopically the liver was normal, so that severe hepatitis and cirrhosis as well as other diseases causing a positive Takata-Ara reaction were ruled out.

TABLE I

TABLE OF PROTEIN VALUES IN CASES OF MULTIPLE MYELOMA

	FIBRIN, GRAMS PER 100 C.C. BLOOD	GLOBULIN, GRAMS PER 100 C.C. BLOOD	ALBUMIN, GRAMS PER 100 C.C. BLOOD	TOTAL PROTEIN, GRAMS PER 100 C.C. BLOOD	PERCENTAGE OF GLOBULIN	ALBUMIN-GLOBULIN RATIO	TAKATA-ARA REACTION
Case 1	0.60	8.40	3.21	12.22	79%	0.38	Unusually heavy precipitate. Two to three times usual amount in each tube.
Case 2	0.78	5.25	4.55	10.58	51%	0.84	Heavy precipitate; two times usual amount in all tubes but the last one.
Case 3	0.42	5.26	3.75	9.41	56%	0.71	Heavy precipitate in each tube. Two times the normal amount in second, third, and fourth tubes.

The values given represent the average of two or more determinations in each case.

In a similar manner the diagnosis in Case 2 was first suggested by a strongly positive Takata-Ara reaction. A biopsy of bone taken in this instance showed the patient to have multiple myeloma. Several determinations of blood proteins gave an average value of 10.58 gm. per 100 c.c. for the total protein; the fibrin was 0.78 gm. per 100 c.c., the globulin 5.25 gm. per 100 c.c., and the albumin 4.55 gm. per 100 c.c. After a diagnosis was established, the patient left the hospital and was lost for further observation.

A definite diagnosis was not established in Case 3 because of the refusal of the patient to complete the hospital study. However, the clinical picture and x-ray examination suggested a multiple myeloma. Here again, a strongly positive Takata-Ara reaction led to the determination of a total protein which was found to be 9.6 gm. per 100 c.c. Two blood determinations gave an average of 9.41 gm. per 100 c.c., for total protein, albumin 3.75 gm. per 100 c.c., globulin 5.26 gm. per 100 c.c., and an albumin-globulin ratio of 0.71.

DISCUSSION

After it was evident that high blood protein values gave such a strongly positive Takata-Ara reaction, it was interesting to speculate whether this threw any new light on the mechanism of a positive Takata-Ara reaction. The first thing suggested was that the flocculated material was Bence-Jones protein, since this protein is commonly believed to be associated with multiple myeloma. However, a careful and repeated search in both blood and urine failed to reveal Bence-Jones protein in any of these cases. Cantarow[3] mentioned that Bence-Jones protein was only rarely found in the cases of hyperproteinemia reported in the literature. Freund and Magnus-Levy[16] also have pointed out that cases passing much Bence-Jones protein in the urine show low or normal total blood protein. So it is evident that this unusual protein is not necessary for either a positive Takata-Ara reaction or hyperproteinemia.

Fibrinogen is occasionally elevated in multiple myeloma with high blood protein values and was found so in these three cases. However, high fibrinogen values can be readily excluded as a cause of a positive Takata-Ara reaction because the test is performed on serum after the fibrinogen is lost with the clots. Specimens of clear serum from these cases gave negative values for fibrinogen.

The exact mechanism of a positive Takata-Ara reaction is not entirely clear but it is believed[10] that an increase in globulin or a decrease in albumin (while perhaps not the only factors) favor a positive Takata-Ara reaction.

It is interesting to compare the total blood proteins and percentage of globulin of cases of advanced portal cirrhosis (where the Takata-Ara reaction is almost always positive) and the total proteins and percentage of globulin in cases of hyperproteinemia. The figures reported by Myers and Keefer[17] for portal cirrhosis can be taken as representative values. In a series of 16 cases the average figure for total blood proteins was 5.3 gm. per 100 c.c. while the globulin averaged 3.1 gm. per 100 c.c., or 58 per cent of the total protein. The average total blood protein of eighteen cases of hyperproteinemia collected from the literature by me was 11.31 gm. per 100 c.c., while the average globulin was 7.94 gm. per 100 c.c., or 70 per cent of the total protein. Gros,[1] in a similar study, reported finding 55 to 60 per cent of globulin in his cases of portal cirrhosis, with an average total protein of 6.3 gm. per 100 c.c., while the total protein of his cases of myeloma averaged 10.8 gm. per 100 c.c. with 76 per cent globulin. Gros, in addition, made further studies of the amount of euglobulin and pseudoglobulin I and II in the globulin fraction of the protein. He found that in portal cirrhosis the globulin fraction was composed primarily of euglobulin and pseudoglobulin I, whereas in multiple myeloma the marked increase in the globulin fraction was due to both pseudoglobulin I and II, while the euglobulin remained normal.

In contrast to the results given by Gros for his myeloma cases are those of Perlzweig and others,[18] Johansen,[19] and Bannick and Greene,[20] who found high values for euglobulin and less marked increases in the pseudoglobulin I and II fractions. Reimann[4] found a fibrinogen of 5.48 gm. per 100 c.c. with a globulin of 3.84 and an albumin of only 0.90 gm. per 100 c.c. Shirer and

others,[21] Cantarow[3] and Jacobson,[22] on the other hand, found the high protein values in their cases to be due to Bence-Jones protein. It is thus evident that high protein values in multiple myeloma may be due to an increase in euglobulin, pseudoglobulin I, pseudoglobulin II, fibrinogen or even Bence-Jones protein.

The results obtained by Gros[1] seem to indicate that the Takara-Ara reaction should be positive in any case where the high total protein is due to an increase in one of the globulin fractions. Evidence seems to favor that the protein increase in most of the instances of hyperproteinemia in multiple myeloma is in one of the usual globulin fractions, since in only four instances has the abnormal increase been due to any other fraction of the total protein. Further studies will undoubtedly show how the Takata-Ara reaction will react when the increase is either in the fibrinogen fraction or due to the presence of Bence-Jones protein. In the present series of cases subdivision determinations of the globulin fraction were not attempted, so it is impossible to say how they compare with the instances mentioned.

It can be readily seen that in cases of hyperproteinemia not only is the globulin fraction more than twice as great as the globulin fraction of the cirrhosis cases, but the globulin percentage (70 to 76 per cent) is noticeably greater than the average globulin percentage (58 per cent) noted in the cirrhosis cases.

Since the precipitate formed in a positive Takata-Ara reaction is believed to be due in part to an increase in globulin and a diminution in the percentage of albumin fraction of the total blood protein, it is understandable why a heavier precipitate should appear in the hyperproteinemia cases than in the cirrhosis cases.

While the three observations recorded in this paper, and the two by Gros do not make an impressive series, they seem to point to the value of the Takata-Ara reaction in detecting at least those instances of hyperproteinemia where the high values are due to the usual globulin fraction. Because of the rarity of this condition, the final opinion will have to rest on statistics collected from scattered sources.

SUMMARY

It was shown by this study that the Takata-Ara reaction can be used to detect the hyperproteinemia due to multiple myeloma where the increase is in the globulin fraction. Because of the simplicity of this test it can be used in a routine fashion where this condition is suspected. If the test is positive, a more detailed study of the blood proteins is indicated. It is not known at present how the Takata-Ara reaction will react when the hyperproteinemia is due either to high fibrinogen or to Bence-Jones protein. The need for further study on this phase of the subject is stressed.

NOTE.—Since this paper was completed, the following significant articles have been published: Salvesen (Acta med. Scandinav. **86**: 127, 1935) reported that hyperproteinemia was a constant feature in each of four cases of Sarcoid of Boeck, and that the increase in each case was in the globulin fraction. The

Takata-Ara reaction was reported as positive. Williams and Gutman (Proc. Soc. Exper. Biol. & Med. **34**: 91, 1936) found that the majority of cases of lymphogranuloma inguinale showed hyperproteinemia with the increase primarily in the globulin fraction. The Takata-Ara reaction was found to be positive in the instances where it was tried. These observations add two more diseases to be considered when hyperproteinemia is discovered, as well as further to show that the Takata-Ara reaction is helpful in detecting hyperproteinemia when increase is in the globulin fraction.

REFERENCES

1. Gros, W.: Zur Frage gestzmassiger Veranderungen des Bluteiweissbildes beim Multiplen Myelom, Deutsches Arch. f. Klin. Med. **177**: 461, 1935.
2. Wintrobe, M. M., and Buell, M. V.: Hyperproteinemia Associated With Multiple Myeloma, With Report of Case in Which Extraordinary Hyperproteinemia Was Associated With Thrombosis of Retinal Veins and Symptoms Suggesting Raynaud's Disease, Bull. Johns Hopkins Hosp. **52**: 156, 1933.
3. Cantarow, A.: Bence-Jones Proteinemia in Multiple Myeloma, Am. J. M. Sc. **189**: 425, 1935.
4. Reimann, H. A.: Hyperproteinemia as a Cause of Autohemagglutination. Observations in a Case of Myeloma, J. A. M. A. **99**: 1411, 1932.
5. Foord, A. G.: Hyperproteinemia, Autohemagglutination, Renal Insufficiency and Abnormal Bleeding in Multiple Myeloma, Ann. Int. Med. **8**: 1071, 1934-35.
6. Sweigert, C. F.: Multiple Myeloma With Hyperproteinemia, Am. J. M. Sc. **190**: 245, 1935.
7. Takata, M.: Ueber eine Kolloidchemische Sero-Diagnostik der Lungenentzundung, Trans. Sixth Congress Far Eastern Assn. Trop. Med. **1**: 693, 1925.
8. Takata, M., and Ara, K.: Ueber eine Neue Kolloidchemische Liquorreaction und ihre Praktischen Ergebnisse, Ibid. **1**: 667, 1925.
9. Staub, H.: Ueber funktionelle Leberdiagnostik, Schweiz. med. Wchnschr. **59**: 308, 1928.
10. Jezler, A.: Beiträge zur funktionellen Leberdiagnostik, Ztschr. f. klin. Med. **3**: 48, 1929.
11. Tannenholz, H.: The Takata Serum Reaction as a Diagnostic Aid in Syphilology and Dermatology, Am. J. Syph. **17**: 352, 1933.
12. Crane, M. P.: A Modified Mercuric Chloride Reaction (Takata-Ara) in Cirrhosis and in Neoplasms of the Liver, Am. J. M. Sc. **187**: 705, 1934.
13. Heath, C. W.: The Takata-Ara Test in the Diagnosis of Liver Disease, New England J. Med. **211**: 1077, 1934.
14. Ragins, A. B.: The Value of the Takata and Ara Reaction as a Diagnostic and Prognostic Aid in Cirrhosis of the Liver, J. Lab. & Clin. Med. **20**: 902, 1935.
15. Magath, T. B.: The Takata-Ara Test of Liver Function, Proc. Staff Meet. Mayo Clinic **10**: 493, 1935.
16. Freund, R., and Magnus-Levy, A.: Multiple Myelome; über Besonderheiten der Blutzusammensetzang, Ztschr. f. klin. Med. **121**: 1, 1932.
17. Myers, W. K., and Keefer, C. S.: Relation of Plasma Proteins to Ascites and Edema in Cirrhosis of the Liver, Arch. Int. Med. **55**: 349, 1935.
18. Perlzweig, W. A., Debrue, G., and Geschickter, C. F.: Hyperproteinemia Associated With Multiple Myeloma, J. A. M. A. **90**: 755, 1928.
19. Johansen, A. H.: Hyperproteinemia and Multiple Bone Tumors, Hospitalstidende 74: 562, 1931.
20. Bannick, E. G., and Greene, C. H.: Renal Insufficiency Associated With Bence-Jones Proteinuria. Report of Thirteen Cases With a Note on the Changes in the Serum Proteins, Arch. Int. Med. **44**: 486, 1929.
21. Shirer, J. W., Duncan, W., and Haden, R. L.: Hyperproteinemia Due to Bence-Jones Protein in Myelomatosis, Arch. Int. Med. **50**: 829, 1932.
22. Jacobson, V. C.: Multiple Myelomata, J. Urol. **1**: 167, 1917.

MACROSCOPIC METHODS IN THE BIO-ASSAY OF PROLAN HORMONES*

S. E. Owen, Ph.D., and L. H. Prince, M.D., Hines, Ill.
With the Technical Assistance of Mr. Q. Polanco

A KEY to the easy interpretation of the macroscopic changes in ovaries of mice reacting to prolan hormones extracted from the urine of patients with teratoma testis is presented. It has proved its value as a time conservator in our laboratories.

The early diagnosis and prognosis of teratoma testis by prolan bio-assays has been previously reported by Zondek,[1] Ferguson,[2] Owen[3] and others. Previous workers have employed the rather tedious method of interpretation of the microscopic findings in ovarian sections from the mouse. We do not now find this procedure necessary.

The details of the technic relative to extract preparation, manner of making injections and the dosages for the various laboratory animals have been reported fully by Zondek (loc. cit.), Cutler and Owen[4] and others. Repetition is not necessary.

There are conditions other than teratoma in the male which occasionally give positive urinary prolan assays. We have found, however, that steadily increasing prolan outputs as determined by serial assays on male urine have invariably been associated with untreated teratoma testis, or with chorion-epithelioma testis.

TECHNICAL CONSIDERATION

Aside from the adoption of the macroscopic findings, we are also now reporting the daily prolan output when the twenty-four-hour urine volume is known. This result is of more significance than the mouse units per liter figure, although both are noted on the laboratory report sheet whenever possible. Since the prolan output varies but little from day to day, regardless of the daily urinary volume, the mouse units per liter figure may be misleading. This is especially so in the summer months when there is a greater variation in the daily urinary output. This dilution factor, we have found, if ignored, may lead to erroneous conclusions.

In Table I some findings are presented. Cases 1, 4, and 9 show per liter and daily output figures in fair agreement as to a true hormone increase. These findings indicate the presence of active teratoma tumor in these cases. The findings were confirmed clinically. Case 2 illustrates a true decrease in

*From the Tumor Research Unit and the Pathological Laboratories, Veterans' Administration Facility.

Received for publication, March 24, 1936.

Published with the permission of the medical director of the Veterans' Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the authors.

urinary prolans following irradiation treatment. The decrease here is reflected in both the mouse units per liter figure and in the total daily output of prolan. This is the usual finding following treatment. A similar decrease is shown for Case 3. The usual dilution is shown in Cases 5 and 7 while greater dilution is illustrated in Cases 6 and 10. In Case 10 the per liter assay figure of two tests is the same, but due to the greater dilution in the second test a true increase of prolan output is displayed. In Cases 8 and 11 fluids other than urine were used for the assay. The chest fluid of Case 8 gave a finding of 30,000 mouse units per liter; this finding prompted the report that this patient had a far-advanced teratoma tumor with undoubted metastases. While subsequent assay on the urine from this patient gave lesser amounts of the hormone, autopsy findings confirmed the laboratory indications as extensive metastases were found, the tissue showing a typical teratoma picture histologically. The hydrocele fluid in Case 11 being negative there were no indications of teratoma tumor.

TABLE I

SHOWING VARIATIONS IN THE PER LITER AND THE TWENTY-FOUR-HOUR URINARY OUTPUT OF PROLAN AND OTHER DATA. ASSAYS IN MOUSE UNITS

PATIENT	ASSAY DATE	URINE VOL. 24 HOUR C.C.	PER LITER ASSAY	TOTAL ASSAY	REMARKS
1	11/ 8/35	1520	100	152	True increase
	12/ 4/35	1500	400	600	
2	11/19/34	1875	400	750	True decrease result of treatment
	2/ 1/35	2000	125	250	
	3/ 2/35	1800	Neg.	Neg.	
3	12/ 2/35	1800	500	900	True decrease
	1/ 2/36	2400	100	240	
4	10/23/35	2000	200	400	True increase
	1/13/36	1500	400	600	
5	1/30/36	2150	200	430	True dilution
6	1/ 4/36	4300	200	960	Great dilution
7	1/29/36	1640	500	820	Normal dilution
8	1/24/36		Chest fluid used, 30,000 M.U. per liter		
	1/29/36	600	10,000	6000	Terminal case
9	1/11/36	1900	200	380	True increase
	2/ 5/36	1250	400	500	
10	10/23/35	2860	200	572	True increase
	1/ 4/36	4800	200	960	
11	1/ 8/36	Hydrocele fluid	100-?	?	Not teratoma
	1/27/36	Hydrocele fluid	Neg.	Neg.	
12	5/17/34	1620	400	648	Not teratoma
	8/23/34	1550	400	620	

In two cases of our series orchidectomy apparently resulted in a steady hormone output, due possibly to a hormone imbalance and not to remaining teratoma tissue. Case 12 illustrates such an incidence. Positive findings up to 400 mouse units of prolan per liter of urine have been noted in these two cases of nonteratoma testicular involvement. Both lesions were extensive enough to completely destroy one testicle. Histologically one of these specimens was an extensive gumma and the other a destructive cyst. Serial prolan

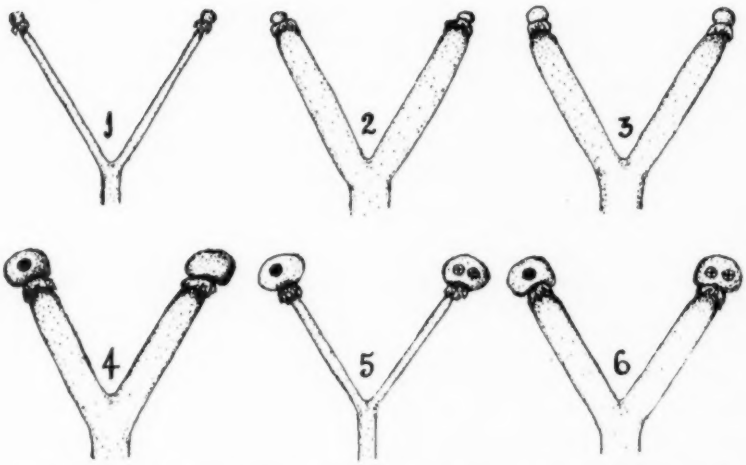

Fig. 1.—Showing the macroscopic findings of the assay as determined from over five hundred tests. (*1*) Shows a negative ovary and negative tube, read as negative. (*2*) A negative ovary but positive tube reaction, read as questionable or slight positive. (*3*) Positive tube and slight ovary effect, read as positive or reaction A. (*4*) Positive tubes and positive ovaries with presence of corpus hemorrhagica, read as reactions A and B. (*5*) Negative tubes but positive ovaries with corpus hemorrhagica and corpus luteum, read as reactions A, B, and C. (*6*) Positive tubes and positive ovaries with corpus hemorrhagica and corpus luteum, read as reactions A, B, and C strong.

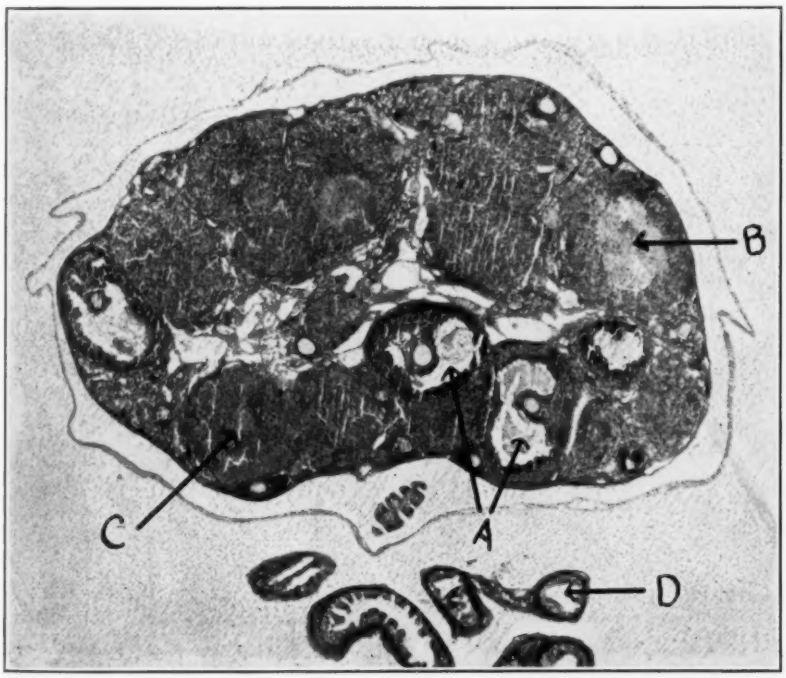

Fig. 2.—Illustrating a composite microscopic finding in a case of teratoma testis. *A*, Ripened graafian follicles with cumulus oophorus. *B*, Hemorrhagic follicle, showing start of luteinization. *C*, Luteinized follicle, corpus luteum. *D*, Hyperemic tube.

assays were positive but did not show increases in prolan output. Teratoma was thus ruled out in both cases and the later histologic data confirmed the findings. Untreated teratoma is invariably associated with a constantly increasing urinary prolan output.

The geographic distribution of approximately 145 patients with teratoma testis, in conjunction with the mail follow-up system employed, occasionally makes the accurate determination of the total daily output of hormones difficult. This is easily obviated when the patients are hospitalized and an accurate measurement of the daily urine is possible.

SUMMARY

1. A key is presented which simplifies reading the prolan bio-assays as conducted on urines from patients with teratoma testis.

2. The importance of determining the daily prolan output, rather than the amount of hormones per liter of urine is stressed.

3. Illustrative comparison between the macroscopic and the microscopic findings on mouse ovaries is made.

4. A means of differentiation of teratoma from other conditions which occasionally give positive prolan assays is presented.

5. The key might be equally adaptable to pregnancy tests.

REFERENCES

1. Zondek, B.: Maligne Hodentumoren und Hypophysenvorderlappenhormone. Hormonale Diagnostik aus Harn, Hydrocelenflussigkeit und Tumorgewebe, Klin. Wchnschr. 11: 274, 1932.
2. Ferguson, R. S.: Quantitative Behaviour of Prolan A in Teratoma Testis, Am. J. Cancer 18: 269, 1933.
3. Owen, S. E.: The Biologic Diagnosis of Teratoma Testis, J. Lab. & Clin. Med. 20: 296, 1934.
4. Cutler, M., and Owen, S. E.: Clinical Value of Prolan A Determinations in Teratoma Testis, Am. J. Cancer 24: 318, 1935.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

POLIOMYELITIS, Toxicity of Organs of Animals Dying After Injections of Emulsions of Stools of Patients With, Toomey, J. A. Am. J. Dis. Child. **50**: 1173, 1935.

Emulsions made of the spinal cords of animals that died after the subcutaneous injection of emulsions made of stools of patients with poliomyelitis were carried in series and were found to be toxic to the fifteenth experimental generation.

Gram-negative bacilli were isolated from every emulsion made of the spinal cord of an animal that died after the subcutaneous injection of an emulsion made of stools of a patient with poliomyelitis.

The predominating organism present in the intestinal contents of normal animals was a gram-positive bacillus. In the animals into which an emulsion had been injected it was a gram-negative bacillus.

The organs of the animals that died after an injection ranged in toxicity as follows: small intestine, spinal cord, liver, kidney, large intestine, spleen, adrenal gland, lung and heart.

BCG, Effect of Vaccination With, in Infancy and Childhood, Aronson, J. D., and Dannenberg, A. M. Am. J. Dis. Child. **50**: 1117, 1935.

Of 41 children who remained in contact with persons having tuberculosis whose sputum contained tubercle bacilli and were vaccinated within the first ten days of life by the peroral administration of BCG vaccine, one (2.4 per cent) died of tuberculosis, while of 84 unvaccinated children living under comparable conditions, ten (11.9 per cent) died from the disease. Of 15 children who remained in contact with persons with tuberculosis whose sputum did not contain tubercle bacilli and were vaccinated with the BCG vaccine, none died of tuberculosis; of 45 unvaccinated children living under comparable conditions, two (4.4 per cent) died of tuberculosis. No deaths from tuberculosis occurred in the children studied who were not exposed to persons known to have manifest tuberculosis, either among the 14 children vaccinated with BCG or among the 38 unvaccinated ones.

Of children in contact with tuberculous persons whose sputum contained tubercle bacilli, 81.6 per cent of 38 vaccinated children and 78.5 per cent of 79 unvaccinated children reacted positively to an intracutaneous injection of tuberculin into the skin. Among fourteen vaccinated and 44 unvaccinated children in contact with persons having tuberculosis whose sputum did not contain tubercle bacilli, 92.8 per cent of the vaccinated and 36.4 per cent of the unvaccinated children reacted to tuberculin. Among children who were not in contact with persons known to have tuberculosis, 75 per cent of eight vaccinated and 23.7 per cent of 38 unvaccinated children reacted to tuberculin.

Of children living in contact with tuberculous persons whose sputum contained tubercle bacilli, 16.6 per cent of 36 children vaccinated with BCG and 56.6 per cent of eighty-three unvaccinated children present roentgenologically demonstrable lesions in the lungs. Among the children living in contact with persons with tuberculosis whose sputum did not contain tubercle bacilli, no pulmonary lesions demonstrable by the roentgen rays were observed in the 13 who had been vaccinated, but 5 per cent of the forty unvaccinated children presented roentgenologic evidence of pulmonary lesions. No lesions demonstrable by means of the roentgen rays were found in the eight vaccinated or 36 unvaccinated children not in contact with persons known to have tuberculosis.

These results indicate that the administration of BCG vaccine to newborn children exposed to patients with manifest tuberculosis may prove of value in reducing the mortality from this disease in infancy and childhood.

The BCG culture used for vaccination in this study has remained attenuated for both human beings and guinea pigs when maintained according to the method prescribed by Calmette.

ICTERUS INDEX, in the Newborn Infant, Bonar, B. E. Am. J. Dis. Child. **50**: 1143, 1935.

The capillary method is satisfactory for routine determinations of the icterus index of the blood of newborn infants.

Hyperbilirubinemia exists at birth and continues through the first twelve days of life regardless of the presence of clinical jaundice, which may not be detected until the index is close to 30.

The icterus index is uninfluenced by sex, loss in weight, coagulation or bleeding time, duration of labor or type of delivery. Low indexes are found in infants weighing above normal at birth, with no jaundice and a high hemoglobin or erythrocyte level.

Certain observations indicate that possibly destruction of blood is not the sole factor in producing neonatal hyperbilirubinemia.

The inherent tendency for the blood of newborn infants to hemolyze is not due to the presence of bile salts. They tend to protect against it.

A brief record of 3 infants born with jaundice is appended.

ERYTHROBLASTOSIS, In the New-Born, Andrews, H. S., and Miller, A. J. Am. J. Dis. Child. **50**: 673, 1935.

Four cases of erythroblastosis in which two of the patients recovered and one came to autopsy are reported. Two of the patients were Negroes; these are the first cases of the disease to be reported in the Negro race. The incidence was 1 in 490 in a review of 1,958 births. Three patients had a normal period of gestation and one was premature. Two were first-born. All were girls. In one case there was a familial history of jaundice, and all the patients were jaundiced during the course of the disease. Hemorrhages appeared in two. The spleen was enlarged in all the patients, and the liver was enlarged in two. There was anemia in all, but no red blood cell count was below 2,000,000. There were many nucleated red cells and a tendency toward leucocytosis, the latter condition disappearing in the patients who recovered. Cells of the myeloid series varied from 1 to 8 per cent, and the color index was near 1 in all cases.

Postmortem examination revealed many hematopoietic centers in the liver, kidneys, spleen and pancreas. Hemorrhages were numerous throughout the body, especially in the lungs. In the kidneys, lungs, and skin were large atypical epithelial cells, an explanation of which is lacking. The bone marrow was moderately hyperactive.

CONJUNCTIVAL FLUID, Quantitative Examination of, in Preparation for Intra-Ocular Operations, Johns, F. M. Am. J. Clin. Path. **5**: 428, 1935.

A capillary pipette 1 mm. outside diameter made from 4 to 5 mm. glass tubing was found to give an average column height of 10 cm. with 0.02 c.c. of fluid. By bending the last few centimeters at right angles, rounding the tip in a small flame, marking a one-half centimeter distance mark from the tip, and the use of rubber tubing equipped with throttle pipette and mouthpiece for suction, an instrument was devised for obtaining approximately 2 c. mm. of fluid for inoculation of media or for obtaining a somewhat larger quantity of fluid to mount on a hemacytometer for cell counts. The butt end of the pipette was plugged with cotton and it was dry sterilized.

In practice the lacrimal sacs are evacuated into the conjunctival sac with gentle pressure. Massage over the eyelids serves to average the fluid contents and tends to desquamate loosened epithelial cells. The lower lid is retracted and the capillary touched to the lower portion of the fold next to the sclera and filled to the mark with fluid. This is ejected onto the surface of a blood agar plate and spread about by the bent end of the pipette. Another quantity of fluid is obtained and mounted directly on a Bass-Johns

hemacytometer which is so constructed that any portion of an entire cubic millimeter of fluid may be counted directly without using a factor. Dilution of the fluid by increased lacrimation does not occur with gentle handling of the pipette.

By these quantitative means it was first determined that the conjunctival fluid of 54 normal young adults contained from 1 to 250 leucocytes per c. mm., with an average of thirty-five cells.

HEMATOLOGIC STANDARDS, Normal, Osgood, E. E. Arch. Int. Med. 56: 849, 1935.

Studies of the erythrocyte count, hemoglobin content, hemoglobin coefficient, cell volume, volume coefficient, color, volume and saturation indexes, reticulocyte count, total and differential leucocyte counts and sedimentation rate for more than 500 persons representing both sexes and all ages from four to over thirty years are presented.

The results of these studies are summarized in charts.

The normal hematologic standards given in a table should be used as a basis for interpretation until they are superseded by more extensive data of equal or greater accuracy.

RENAL THRESHOLD For Glucose in Man, Sherrell, J. W., and Mackay, E. M. Arch. Int. Med. 56: 877, 1935.

These experiments and our consideration of the factors involved in the determination of the renal threshold for dextrose lead us to the conclusion that the threshold as a "normal figure" is a misnomer. That a given subject has his own particular level above which sugar appears in the urine is probable, provided the conditions are the same each time. Under ordinary circumstances the figure found simply represents the situation as it exists at that particular moment. A normal average for the renal threshold should never be given, for it may be misleading.

For practical purposes the renal threshold for dextrose is of clinical interest only when dextrose is detected in the urine, for this indicates either hyperglycemia or a so-called "renal diabetes." In the absence of sugar in the urine qualitatively the renal threshold has no place.

DEXTROSE TOLERANCE CURVES, Interpretation of Abnormal, Occurring in Toxemia in Terms of Liver Function, Soskin, S., Allweiss, M. D., and Mirsky, I. A. Arch. Int. Med. 56: 927, 1935.

The abnormal dextrose tolerance curves occurring in cases of toxemia are due to the effects of the toxin on the liver and not on the pancreas.

The toxin interferes with the homeostatic mechanism of the liver which we have described, i.e., the mechanism by which the liver decreases its supply of blood sugar in response to an influx of exogenous sugar.

The interpretation of abnormal dextrose tolerance curves in terms of the function of the liver, whether such tests occur in toxemia, diabetes or other conditions, relates all such curves to a common underlying mechanism instead of to various factors as formerly. This not only renders the curves themselves more intelligible but gives a more rational view of "insulin resistance," the use of carbohydrate therapy and other related phenomena.

BLOOD: A New Hematological Stain, Kingsley, D. M. Stain Techn. 10: 127, 1935.

The stain proposed by the author is quickly and simply prepared by mixing equal parts of the following permanent stock solutions:

Solution I

Methylene blue, U.S.P., med.	0.0650	g.
Methylene azure A	0.0100	g.
Glycerin, C.P.	5.0	c.c.
CH_3OH C.P.	5.0	c.c.
Distilled water	25.0	c.c.
Buffer, pH 6.9	15.0	c.c.

Solution II

Methylene violet (Bernthsen)	0.0130	g.
Eosin, yel., w. s.	0.0450	g.
Glycerin, C.P.	5.0	c.c.
CH_3OH, C.P.	10.0	c.c.
Acetone, C.P.	35.0	c.c.

The mixed stain is usable for at least eight months, and is applicable to practically all hematologic purposes: blood smears, fixed sections, frozen sections, and touch preparations. In the technics utilized to produce its action on preparations treated in different ways, the only variants are the methods for treating the cells, while the stain itself remains totally unchanged for all purposes. For blood smears fixed with methyl alcohol, the technic consists merely of pouring the stain on the slide, leaving it for five to seven minutes, and then washing it off. On sections, a further process of differentiation with acid acetone is rapidly carried out. The various types of granules, including megakaryocyte and platelet granules, are clearly demonstrated. For frozen sections, the technic is extremely rapid, yet yields excellent differentiation.

The technic for sections is as follows:

a. Paraffin sections

1. Xylol.
2. Descending alcohols (70 per cent alcohol with iodine if necessary).
3. Distilled water.
4. Flood with stain for from nine to ten minutes.
5. Rinse in distilled water (change often).
6. Wash in distilled water acidified with acetic acid, 15 drops (0.75 c.c.) of 1 per cent solution to 60 c.c. of water.
7. Wash in distilled water to stop action of acid and allow examination under the microscope.
8. Wipe off excess water and rinse in C.P. acetone to which has been added for every 60 c.c. 10 drops (0.5 c.c.) of 1 per cent acetic acid and also 0.01 c.c. of 5 per cent eosin in CH_3OH (or 0.005 g.).
9. Rinse in C.P. normal butyl alcohol to which has been added, for every 60 c.c., 0.02 c.c. of 5 per cent eosin in CH_3OH (or 0.001 g.).
10. Rinse in neutralized xylol. Examine under microscope for proper differentiation. If satisfactory, go on with (11). If not, go back through (9), (8), and even (6) if necessary. Then as above again.
11. Three changes of neutralized xylol (change often).
12. Mount in neutralized xylol-dammar and keep from light.

b. Frozen sections

1. Blot to slide.
2. Then as in (4) above.
3. For speed, one may stain only two to three minutes and thereafter examine in water, without differentiation. Staining over five minutes is rarely required on unfixed sections. Only one rinse in xylol is necessary for slides not to be kept permanently, and (6) above may be omitted.

DICK TEST, Comparison of Intracutaneous and Subcutaneous, Spivek, M. L. Am. J. Dis. Child. **50**: 1113, 1935.

Intracutaneous and subcutaneous injections of 0.1 c.c. of toxin for the Dick test, each performed in duplicate, gave identical reactions in 636 (97.4 per cent) of 653 children.

The reactions obtained in 17 tests (2.6 per cent) did not agree. Of these there were 14 positive reactions to subcutaneous injections and 3 to intracutaneous injections with negative reactions in the duplicate tests.

There was agreement in all but 6 instances as to the degree of erythema noted.